Endocytosis in trypanosomes occurs via the flagellar pocket. Macromolecules enter the flagellar pocket from the outside of the cell and are then taken up by endocytosis. This picture shows particles of colloidal gold inside the flagellar pocket prior to endocytosis. Magnification: 80,000 ×.

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Foreword

The International Laboratory for Research on Animal Diseases (ILRAD) has recently developed interests in several areas of cell biology and biochemistry that are relevant to trypanosomiasis and theileriosis (East Coast fever [ECF]). The reasons for this interest stem from a perceived difficulty of tackling trypanosomiasis by conventional vaccination procedures due to the tremendous antigenic variation that trypanosomes can undergo. Thus, approaches other than a purely immunological one are now being explored. As a part of this new emphasis, a four-day workshop was held at ILRAD to review the current state of knowledge in the field of protein traffic and catabolism and to attempt to address these issues, with particular reference to parasitic diseases. The topics covered included such areas as protein targeting, protein assembly, protein degradation, endocytosis and lysosomal activity. There were 20 speakers from Europe and America and 8 from ILRAD, as well as approximately 40 observers from ILRAD and other research organizations in Africa. The organizers of this workshop would like to thank the United Nations Development Program (UNDP) for their financial support, and the distinguished visitors for their most valuable scientific contributions, which helped to make the meeting a success.

C. Redman (New York Blood Center) gave an elegant exposition on how a relatively simple procedure such as SDS-polyacrylamide gel electrophoresis (SDS-PAGE) can be used to delineate the steps involved in such complex tasks as protein macromolecular assembly and disulphide cross-linking. The role of disulphide bond formation in protein assembly and targeting was also addressed by T. Morrison (University of Massachusetts Medical School) who showed that, while virion associated protein was exclusively in disulphide-linked oligomers, not all haemoglobin-nitrogen (HN) protein (in the Newcastle disease virus) was in this form during processing from the endoplasmic reticulum (ER). The importance of understanding such assembly process was exemplified by W. Fish (ILRAD), who showed that the immunological exposure of the species cross-reactive epitope, called the cross-reacting determinant (CRD), of the variant specific glycoprotein (VSG) of *Trypanosoma congolense* could be dissected into several distinct enzymatic and non-enzymatic (disulphide-bond reduction) steps. Apparently the enzymatic removal from VSG of the proposed membrane anchor, dimeristoylphosphatidyl-inositol, is not a pre-requisite for CRD exposure as formerly believed. Indeed, D. Grab (ILRAD) provided evidence that a phospholipase-C, which had been proposed to mediate the release of the surface coat from the parasite, was probably an intracellular enzyme, thus also raising doubts as to the proposed physiological role of the enzyme as a mediator for the release of VSG from the surface of the parasite.

The importance of correctly folded proteins was further explored by A. Goldberg (Harvard), who described the role of cytosolic proteases that degrade abnormally synthesized proteins and proteins that have been damaged by other processes such as the action of free radicals. Free radicals and their reaction products have long been recognized as agents capable of reacting with lipids and carbohydrates, but only recently was evidence found to show that proteins may also be affected. Interestingly, R. Dean (Brunei) showed that the bloodstream forms of African trypanosomes, which contain the thick VSG coating, are apparently more susceptible to free radical damage than are the same parasites from which the coat has been removed enzymatically, or those that have lost it (procyclic forms) in the normal course of the parasite life cycle! Such observations emphasize the importance of ascertaining the role of free radical damage to cellular proteins.
The purification and characterization of lysosomal enzymes from *Entamoeba histolytica* (A. Barrett, Strangeways), and African trypanosomes (J. Lonsdale-Eccles, ILRAD) were described and their biochemical properties shown to be distinct from mammalian thiol-dependent cathepsins. The possibility that such differences between mammalian and parasite enzymes may be exploitable was addressed by E. Shaw (Freidrich Miescher Institute, Basel), who described a variety of peptidyl derivatives that acted as specific affinity-labelling inactivators of cysteine and serine proteinases. By appropriately tailoring the inhibitor, he was able to demonstrate the intracellular inhibition of different lysosomal enzymes as well as the blockage of intracellular processes such as antigen presentation or proinsulin processing. Interestingly, the trypanolytic activity of human serum could be blocked by lysosomotropic agents such as chloroquine (B. Betschart, Swiss Tropical Institute). J. Kay (University College, Cardiff) also described a modulation of intracellular antigen processing using an inhibitor that had a specificity against aspartyl proteases such as cathepsin E.

R. Kelly (University of California) described how sorting signals, which appear to be conserved in different cells, are involved in deflecting proteins from an otherwise default secretory pathway. K. Simons (European Molecular Biology Laboratory) addressed how such sorting can occur in polarized cells (e.g., Madin-Darby canine kidney cells, which can be infected by vesicular stomatitis virus only on the basolateral side and hepatitis A virus only on the apical side). He eliminated carbohydrate as a possible signal and showed that the lipid/glycolipid gradient from one side to the other is affected by a gate at the tight junctions between cells. By a novel technique (of decapitating the cells!) he was able to show that it is possible to dissect some of the biochemical events that are involved in such sorting. Whether trypanosomes, which are also polarized cells, will be amenable to such studies remains to be determined.

The biological importance of the lysosomal system was beautifully shown by E. Harms (Universitaets-Kinderklinik). He demonstrated the importance of several of the lysosomal enzymes using hereditary deficiencies of the enzymes to show their roles in the maintenance of the viability of the individual. A. Fok and R. Allen (Hawaii) gave detailed descriptions of the endocytotic pathways of *Paramecium*, whose endocytotic system is the best studied of the protozoans. Their digestive process appears to undergo a rapid, complex series of maturation events (approximately 30 minutes from internalization to defecation) that involves fusion of acidic vesicles and ultimate secretion of lysosomal contents. M. Moore (Plymouth, UK) described how lysosomes become destabilized when cells have been in contact with xenobiotics. This increased lysosome fragility is associated with enhanced catabolic activity. Destabilization of the lysosomal membranes can occur with a wide variety of perturbations, including pH. Indeed, using such properties, L. Huang (University of Tennessee) described the use of a pH-dependent drug delivery system that has been successfully employed in transfection and in other studies. Specific targeting of the liposomes is achieved by incorporating appropriate target antibodies in the surface of the liposome.

However, the use of immuno-liposomes will require a detailed knowledge of the intracellular compartments and sorting mechanisms. J. Slot (University of Utrecht), for example, has been studying how the lysosome membrane differs from that of the plasma membrane (he has proposed that sorting may be a consequence of changes in surface area within the organelles). A. Helenius (Yale University) has taken this one step further and suggested that there are two cycling systems, with early lysosomes and late lysosomes recycling their membranes in separate cycles. In the case of trypanosomes, progress in understanding sorting has been made by P. Webster (ILRAD). He has shown that there appears to be several sorting systems that may be effective according to either the chemical structure or the size of the respective compounds that are subject to sorting. This sorting appears to occur in a complex network of interconnected tubules or cisternae rather than in discrete vesicles. In contrast, F. Maxfield (Columbia University) has shown that in mammalian CHO cells, 80% of a
2-macroglobulin goes to lysosomes whereas 90% of transferrin goes to the surface. Each appears to be in different-sized vesicles that are subject to different pH values in the different endocytic pathways. These endosomes fuse actively for several minutes after the initial internalization with a concomitant large increase in the number of non-recycling components but no increase in the recycled components (analogous to fractional distillation). To dissect the endocytic steps, R. Anderson (University of Texas) has begun an ambitious project to study coated pit assembly in vitro. Initial studies suggest that he is able to prepare, in vitro, clathrin-coated pits that have properties similar to the in vivo ones.

During the blood-infective stage of their life cycle, T. brucei can undergo a morphologic differentiation that is accompanied by changes in several protein kinase substrates (Aboage-Kwarteng, ILRAD). These proteins are phosphorylated by cytosolic casein kinase-II-like enzymes that differ from mammalian casein kinase-II. Whereas the kinases from the African trypanosome appear to be involved in the switch from dividing to non-dividing cells, protein kinases in Theileria may act as growth signal transducers in the Theileria-induced-cell proliferation (O. ole-MoiYoi, ILRAD). The Theileria protozoan parasites are unusual in that they induce lymphoblastogenesis and clonal expansion of otherwise quiescent cells. A definitive role for the kinases in this transformation process has yet to be found. Although not causing transformation, plasmodial infection of cells causes morphologic changes in host structure. Some of these, M. Aikawa (Case Western Reserve University) reports, are to allow for the traffic of parasite protein to the host cell surface.
Opening address

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The International Laboratory for Research on Animal Diseases (ILRAD) was established in 1973 to undertake programs of research and training aimed at the improved control of livestock diseases. From the outset the emphasis of the Laboratory has been improved control of the forms of animal trypanosomiasis and theileriosis that continue to plague the livestock industries of many countries in Africa.

Much of our work has been concerned with the antigenicity of the causative organisms and immunological aspects of infection with the aim of developing methods of immunization. However, both disease complexes are caused by pathogenic protozoa, and as you all know, effective vaccines against protozoa are hard to find. In recognition of this, our research programs are broadly based and we take a fairly wide view of the epidemiology, biochemistry and pathology of infections, as well as of immunity.

This week, with your help, we will be concentrating on aspects of the problem presented by tsetse-transmitted trypanosomiasis. This is of particular interest to us and is being tackled at ILRAD in several ways, namely by:

1. The development of improved techniques to diagnose infections, the identification of causal organisms, assessment of their sensitivity to trypanocidal drugs and improvement of therapeutic treatment regimes.

2. Basic research studies on the biology of trypanosomes, particularly their antigenicity, metabolic processes and pathways, and control of differentiation processes.

3. Examination of non-specific and specific resistance to infection, particularly the responses of trypanotolerant livestock.

4. Research on selected aspects of the pathogenesis of infection, particularly the development of anaemia and certain reproductive and endocrine disorders.

Our objective at ILRAD is to identify ways in which we can significantly improve the control of trypanosomiasis as a result of work on the parasites in one or several of these research areas. Other organisations are giving their attention primarily to aspects of tsetse control. During the coming week we will be concerned mainly with work on various aspects of trypanosomal metabolism, especially metabolic and synthetic pathways.

The move to convene this workshop came from two sources, ILRAD and United Nations Development Program (UNDP). It has been ILRAD's practice for some years to organise reviews of aspects of ILRAD's research programs, with contributions from ILRAD scientists and scientists working in the same field at other Laboratories. Three years ago we reviewed bovine immune responses, last year the bovine major histocompatibility complex and trypanotolerance in African livestock. On this occasion the theme is Protein Traffic in Parasites
Enthusiasm at ILRAD for this theme received a special boost late last year from UNDP. For ten years ILRAD has received financial support for the research and training program on African trypanosomiasis from the UNDP global and interregional development program. Late last year, UNDP offered additional support to enable ILRAD to organise workshops to examine recent advances in aspects of cellular and molecular biology of parasitic protozoa that are pathogens of both humans and livestock. The UNDP wish to stimulate dialogue among people interested in medical and veterinary aspects of research on trypanosomiasis and fruitful collaboration between ILRAD and leading centres of excellence around the world.

The theme of this week's activities is therefore very appropriate. Protein traffic in parasites has many features in common with corresponding processes in mammalian cells. Such processes are vital to the survival and multiplication of the parasites and thus may offer potential targets for immunological and chemical attack. We have therefore invited you here as scientists distinguished in cell biology to interact with ILRAD staff members and scientists from other organisations who are working on pathogenic trypanosomases and to ensure that current concepts and findings from mammalian cell biology are available to those working towards improved control of parasites. Specifically, therefore, the objectives of the workshop are:

1. To review findings from research on the biology and biochemistry of mammalian cells and other organisms for their possible relevance and application to research programs on trypanosomiasis and other parasitic diseases.

2. To review research in progress at ILRAD and elsewhere on aspects of the biochemistry and cell biology of pathogenic trypanosomes for its quality and relevance to the potential control of human and animal trypanosomiasis.

3. To identify research areas where new ideas and new findings offer possibilities for developing new research approaches.

4. To encourage and strengthen links among scientists at ILRAD and elsewhere working on topics of interest and relevance to ILRAD's research programs on cell biology and biochemistry.

At the end of the workshop I trust it will be possible to put together a summary of the presentations and discussions in a report for use at both ILRAD and UNDP. It is also desirable that new personal associations and research activities develop in view of the urgent need to alleviate the continuing burden of trypanosomiasis and other protozoan diseases on the livestock and peoples of Africa. I wish you all a most successful and productive meeting.
In the life cycle of African trypanosomes, the parasites differentiate through several morphologically and biochemically distinct forms. In the bloodstream of the mammalian host, *Trypanosoma brucei* differentiates from a rapidly dividing slender form to a non-dividing stumpy form. While it is clear that this differentiation is important to the life cycle and survival of the parasite, the factors that control the processes have not been identified.

The role of protein phosphorylation in both differentiation and transformation of eukaryotic cells is well established. In *Saccharomyces cerevisiae*, cAMP-dependent protein phosphorylation is required for cell cycle initiation.¹ In the fission yeast *Schizosaccharomyces pombe*, the cdc2 gene, whose transcription is required at the start of the cell cycle and probably later in mitosis, encodes a phosphoprotein with protein kinase activity.² Our objective in this study is to identify differentially phosphorylated proteins in bloodstream *Trypanosoma brucei* and to determine their roles in the differentiation from slender to stumpy form parasites. The basis of an experimental approach with a phosphoprotein of unknown function was nicely formulated by Robinson, who said that: "One enters the story in the middle by first targetting a phosphoprotein as a 'band on a gel' and then proceeding both backwards (what protein kinase is responsible for its labelling?) and forwards (what is the protein's function?)". This report describes the identification of two differentially phosphorylated proteins and a partial characterization of the protein kinase(s) that phosphorylate them.

**Methods**

*Preparation of trypanosome cytosolic fraction*

*Trypanosoma brucei* ILTat 1.1 was used in this study. The infection produced in laboratory rodents is sufficiently synchronous to allow 100% slender and greater than 90% stumpy trypomastigotes to be isolated on days 4 and 7, respectively, after infection. Trypanosomes were grown in lethally irradiated rats and the percentage of stumpy forms on different days after infection determined according to Ormerod.⁴ When the desired stage of parasitaemia was attained, the trypanosomes were purified from blood elements by isopycnic Percoll gradient centrifugation⁵ and DEAE-cellulose chromatography.⁶ The purified trypanosomes (5 x 10¹⁰) were suspended in 25 mM Hepes, pH 7.0, 250 mM sucrose, 2 mM EDTA, 10 mM EGTA and 2 mM DTT with 40 μg/mL each of the protease inhibitors leupeptin, antipain and E-64 and disrupted in a French Press at a pressure of 2,500 psi. The homogenate was centrifuged at 100,000 g for 60 minutes to give a cytosolic fraction (HSS) and pellet (HSP).

*Protein kinase assay*

Assays were carried out in a total volume of 150 μl with the following components: 50 mM Tris-HCl
pH 7.2, 150 mM KCl, 10 mM MgCl₂, 5 mM ATP, 10 μCi [γ-32P]ATP (5000 Ci/mmol), 150 μg of protein and enzyme source (usually stumpy HSS). The reaction was carried out on ice for 10 minutes and stopped by the addition of 50 μl 4 x SDS sample buffer (200 mM Tris-HCl pH 6.8, 8% SDS, 40% glycerol and 4% 2-mercaptoethanol) and boiling for 5 minutes.

Electrophoresis

The phosphorylated proteins were separated by SDS polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli, using 7.5-15% polyacrylamide gradient gels. The gels were stained with Coomassie blue, destained and dried. Radioactive protein bands were detected by autoradiography. Quantitative measurement of phosphate incorporation into individual bands were made by counting, in aquasol, the respective regions excised from the gel.

The phosphorylated amino acids recovered from protein bands of approximate Mr 42 and 37, which had been electroeluted from SDS-PAGE gels, were determined using electrophoresis on cellulose thin-layer plates.

Results

Clear differences were observed in the profiles of the cytosoluble (HSS) phosphoproteins of trypanosomes isolated between 4 and 7 days after infection (Figure 1). There is decreased phosphorylation of a protein with Mr 80 kDa (pp80) with the change from the slender to stumpy form parasite. However, all the work described in this paper is on the two proteins, with Mr of about 42 kDa (pp42) and 37 kDa (pp37), which show increased phosphorylation with the differentiation of the slender to the stumpy parasite. Protein kinase assays of the nonsoluble fractions (HSP) show a much lower level of phosphorylation of both pp42 and pp37, which suggests that both proteins are predominantly cytosolic.

Trypanosome protein kinases are able to use both ATP and GTP as phosphoryl donors. Heparin, which inhibits casein kinase II but not casein kinase I, inhibits the phosphorylation of pp42 but has little effect on pp37 phosphorylation. Suramin, a trypanocidal polysulphated naphthylamine, was also found to inhibit the activity of trypanosome protein kinases (50% inhibition at about 25 mM).

The metal ion requirements for the phosphorylation of both proteins suggest the presence of more than one protein kinase in T. b. brucei. The optimum Mg²⁺ concentration for the phosphorylation of pp42 is 10 mM. For pp37, however, the optimal Mg²⁺ concentration is 100 mM. The trypanosome protein kinases can use Mn²⁺ at approximately five-fold lower concentrations than those required for Mg²⁺.

Assays with enzymatic amounts of D7 HSS and a variety of substrates showed that the trypanosome protein kinases can phosphorylate the acidic proteins casein and phosvitin but not the basic histones or protamin (Figure 2). Analysis showed that both pp37 and pp42 are phosphorylated on serine and threonine residues.

Discussion

Protein phosphorylation has emerged as a mechanism involved in the hormonal control of metabolism in mature cells and in the changes in cellular activity associated with cellular proliferation and differentiation. The work presented above demonstrates the coincidental increased phosphorylation of two proteins (pp42 and pp37) with the differentiation of slender bloodstream forms of T. b. brucei into the stumpy forms. Previous studies of T. b. gambiense have shown the presence of multiple cAMP-independent protein kinases, which can phosphorylate phosvitin, protamine and histones. However, no endogenous protein substrates of these kinases were determined. Our work has identified two differentially phosphorylated endogenous proteins in T. b. brucei.

Our studies on the effects of metal ions on the phosphorylation of pp42 and pp37 suggest that the
two proteins are phosphorylated by different enzymes. This is in agreement with studies of *T. b. gambiense* that have demonstrated the presence of multiple protein kinases.\(^{11}\)

It has been shown that phosphorylation on both serine and threonine residues differentiates casein kinase II activity from casein kinase I, which only phosphorylates serine residues. Casein kinase II is also distinguished from casein kinase I in its ability to use both ATP and GTP as phosphoryl donors in phosphotransferase reactions.\(^9\) Our studies with effectors are independent of cyclic-nucleotides, calcium/calmodulin, and calcium/phospholipid. Phosphoamino acid studies on pp42 and pp37 showed that the amino acid residues phosphorylated were serine and threonine, thus implicating the involvement of casein kinase II-like enzymes in these phosphorylations and eliminating the involvement of tyrosine kinases. Further studies established the trypanosome protein kinases as casein kinase II-like because of their ability to phosphorylate casein and phosvitin and their stimulation by KC1.

Sensitivity to heparin inhibition has been used to differentiate casein kinase II from casein kinase I.\(^9\) We observed that while the phosphorylation of pp42 was sensitive to inhibition by heparin, the phosphorylation of pp35 was relatively unaffected. However, recent studies in *Xenopus laevis* oocytes\(^{10}\) and yeast\(^{14}\) have identified heparin-insensitive casein kinase II enzymes. Thus the validity of using susceptibility to heparin inhibition as a criterion for distinguishing of casein kinase II from the type I enzyme is questionable.

The polyamine spermine has been shown to activate casein kinase II.\(^9\) In contrast, we found that the phosphorylation of pp42 was inhibited by spermine while pp37 phosphorylation was largely unaffected. This further suggests that the two proteins are phosphorylated by different enzymes. Another difference between the casein kinase II-like activity of trypanosomes and the enzyme described from mammalian sources is the effect of polylysine and polyarginine. While the classical mammalian enzymes are activated by these effectors, the low-density-lipoprotein-receptor kinase\(^{15}\) and the trypanosome enzymes are inhibited by them. Thus the inhibition studies show that the protein phosphorylation in trypanosomes is inhibited by large negatively and positively charged molecules.

In conclusion, we have identified two differentially phosphorylated proteins in *T. b. brucei*. The proteins with Mr 42 and 37 kDa on SDS-PAGE are more heavily phosphorylated in the stumpy parasite than in the slender. The activities of the enzymes that phosphorylate the proteins are independent of cyclic-nucleotides, calcium/calmodulin or calcium/phospholipids. The trypanosome protein kinases are casein kinase II-like but show some significant differences from the classical mammalian casein kinases.

**References**


Figure 1. Protein phosphorylation of the cytosoluble fraction (HSS) of trypanosomes isolated 4-7 days after infection. The proportions of stumpy-form parasites in the trypanosomes isolated on days 4, 5, 6 and 7 were approximately 0, 10, 70 and 95%, respectively. Three proteins were differentially phosphorylated: a protein with Mr of 80 kDa (pp80) decreased in phosphorylation with increasing proportion of stumpy-form parasites, whereas the other two proteins, with Mr 42 kDa (pp42) and 37 kDa (pp37), showed increasing phosphorylation with an increasing percentage of stumpy parasites.

Figure 2. Trypanosome protein kinases can phosphorylate the acidic proteins casein and phosvitin. The lanes show kinase assays done with 5 mg D7 HSS alone (1), 150 mg D7 HSS (2) and 5 mg D7 HSS + 5 mg/mL casein (3), phosvitin (4), protamine (5), histone 2A (6) and histone VS (7).
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Erythrocyte Changes Induced by *P. falciparum*
Movement of the *P. falciparum* Protein to the Erythrocyte Membrane Resulting in
Lysis of the Erythrocyte and Release of Gametes
Erythrocyte Changes Induced by *P. brasilianum* Infection
Erythrocyte Changes Induced by *P. vivax*
Conclusions
References

Morphological and functional changes occur in erythrocytes infected with malarial parasites. These erythrocyte alterations appear to relate to the capability of malarial parasites to change the properties of the erythrocyte and its membrane in order to export malarial proteins from the parasite to the host erythrocyte membrane. Although the significance of these changes are not clear, they appear to be involved in the development of malaria-related complications in the host.

In my presentation, the host cell alterations induced by primate parasites, including *Plasmodium falciparum*, *P. brasilianum*, *P. vivax* and *P. malariae* infections, will be discussed and the trafficking of these malarial proteins from the parasite to the erythrocyte membrane will be described.

**Erythrocyte Changes Induced by *P. falciparum***

*Plasmodium falciparum* infection induces morphological changes in erythrocytes that include (1) knob-like protrusions of the erythrocyte membrane, (2) clefts in the erythrocyte cytoplasm and (3) electron dense material (EDM) in the erythrocyte cytoplasm.  

Electron dense material and clefts appear to be associated with the formation of knobs. EDM is seen associated with the plasma membrane of intracellular parasites and is also associated with unit membrane-bounded Maurer's clefts. This EDM has the same density and appearance as the material located under knobs at the erythrocyte membrane. Immuno-electron microscopy has demonstrated that EDM and knobs contain *P. falciparum* antigens. Thus, the parasite-derived EDM appears to be transported from the parasite plasmalemma to the erythrocyte membrane via clefts in the erythrocyte cytoplasm.

At least four malarial proteins (HRP1, HRP2, EMP1 and EMP2) have been identified on the surface of *P. falciparum*-infected erythrocytes. Three of these proteins - HRP1, EMP1 and EMP2 - are localized in knobs as demonstrated by immuno-electron microscopy. The presence of histidine-rich proteins in HRP1 is an important factor in knob formation, and a histidine
analogue, 2-fluoro-L-histidine, reduces the formation of knobs and increases the amount and density of EDM. This indicates that this histidine analogue blocks export of EDM from the parasite to knobs, thereby inhibiting knob formation.

The knobs have cytoadherence activity to the endothelial cells and are responsible for sequestration of infected erythrocytes in organs such as the spleen, pancreas, heart and brain. Host cell molecules such as OKMs and thrombospondin have been suggested as endothelial cell surface receptors for the knobs of *P. falciparum*-infected erythrocytes. By acting independently or together, these proteins could play a role in cytoadherence of knobs *in vitro*.

**Movement of the *P. falciparum* Protein to the Erythrocyte Membrane Resulting in Lysis of the Erythrocyte and Release of Gametes**

During erythrocyte invasion by merozoites, a molecule (Pf155/RESA) is injected by the parasite into the erythrocyte membrane and the entire membrane becomes covered with Pf155/RESA. During differentiation of the parasite to the trophozoite stage, the antigens, as detected by immuno-gold labelling, are no longer detectable on the erythrocyte membrane, while gold particles become more numerous within the parasite and in the erythrocyte cytoplasm adjacent to the parasite. As the parasites develop into schizonts, more antigen appears within the parasite and some of it appears to diffuse into the erythrocyte cytoplasm. At the segmented schizont stage, many intraparasitic gold particles are associated with rhoptries and micronemes of developing merozoites.

In addition, Pf155/RESA appeared to be associated with gametocytes. Therefore, using a MAb to Pf155/RESA and rabbit sera to two different repeat peptides of Pf155/RESA, we studied the location of Pf155/RESA after induction of gametogenesis. Five minutes after gametogenesis was triggered, the parasitophorous membrane no longer surrounded the parasites, bringing the parasite membrane in contact with erythrocyte cytoplasm. Clear spaces appeared throughout the haemoglobin-rich host cytoplasm. Pf155/RESA was now localized in the haemoglobin directly surrounding the spaces. No membrane existed between the spaces and the haemoglobin. The spaces with surrounding malarial protein extended to the erythrocyte membrane. After lysis of the erythrocyte membrane, the antigen was distributed along the erythrocyte membrane and throughout the space between the gamete and the erythrocyte membrane.

Our study, therefore, indicates that Pf155/RESA antigen is responsible for disrupting the red blood cell cytoplasm and lysing the erythrocyte plasma membrane, thereby allowing release of *P. falciparum* gametes from their host cells.

**Erythrocyte Changes Induced by *P. brasilianum* Infection**

*Plasmodium brasilianum* is a quartan malarial parasite of New World monkeys. A close evolutionary relationship between *P. brasilianum* and the human malaria parasite, *P. malariae*, is suggested by analogies in their morphology and course of their development in primate hosts.

Erythrocyte changes include the formation of knobs on the infected erythrocyte membrane and clefts in the erythrocyte cytoplasm. The knobs are similar to those seen in *P. falciparum*-infected erythrocytes. The cytoplasmic clefts can be divided into three morphological types, namely, short, long and circular clefts. Recently a series of monoclonal antibodies (MAbs) against blood stages of *P. brasilianum* has been developed that reacts with knobs and clefts as detected by immunoelectron microscopy.
MAbs that recognize an antigen of 38 kDa molecular weight reacted with short clefts. Immuno-gold particles appeared over short clefts and were not associated with long clefts, reflecting the specificity of binding of these MAbs to short cleft antigen. MAbs that recognize an antigen of 16 kDa molecular weight reacted with long clefts and also with the parasitophorous vacuole membrane (PVM). The long clefts appear to be continuous with both PVM and IRBC membranes. Immunoelectron microscopy identified the presence of 14, 16 and 19 kDa malarial proteins in knobs.

The difference in antigenic composition between short and long clefts has not been reported for any plasmodial species. That the long cleft antigen was associated with the PVM and the short cleft antigen was not suggests that different mechanisms incorporate the two antigens into their respective clefts. The clefts have been suggested to function in the transport of knob materials, as demonstrated in *P. falciparum*-infected erythrocytes. However, cleft and knob antigens of *P. brasilianum* are immunologically distinct. It is possible that the nature of antigens being transported via clefts varies with the stage of parasite development and that early in the parasite life cycle, knob proteins are principally transported. At a later stage of development, the antigenic composition of the clefts might change.

The relationship between knob proteins of *P. brasilianum* and *P. falciparum* remains unclear. Although the knobs of *P. falciparum*-infected erythrocytes have cytoadherent activity, those on *P. brasilianum*-infected erythrocytes do not appear to possess cytoadherent functions. Further studies may be required to determine the function of the knobs that appear on the membrane of erythrocytes infected with *P. brasilianum*.

**Erythrocyte Changes Induced by *P. vivax***

Erythrocytes infected with *vivax*-type malaria parasites are characterized by Schuffner's dots, which appear as multiple small brick-red dots in Giemsa-stained thin films. Electron microscopy demonstrated that Schuffner's dots are composed of caveola-vesicle complexes (CVC). This structure consists of caveolae to which vesicles are attached in an alveolar fashion. Another host cell alteration observed within the infected erythrocyte cytoplasm is cytoplasmic clefts.

Recently investigators produced a series of MAbs against various antigens of erythrocytic stages of *P. vivax*. Among them, MAbs that identified a 95 kDa **S**-methionine-labelled *P. vivax* protein produced a stippled pattern similar to Schuffner's dots in *P. vivax*-infected erythrocytes. Other MAbs that reacted with a 28 kDa **S**-methionine-labelled protein gave a linear pattern by immunofluorescent microscopy.

To identify the precise location of *P. vivax* antigens that react with these MAbs, we performed post-embedding immunoelectron microscopy. MAbs against a 95 kDa **S**-methionine-labelled protein gave a pattern similar to that of Schuffner's dots by the immunofluorescence test (IFA). Immunoelectron microscopically specific label was found by immunoelectron microscopy to be associated with vesicles of the CVC, whereas only a few gold particles were associated with the caveolae. Vesicles scattered throughout the erythrocyte cytoplasm were also labelled with gold particles.

Other MAbs that react with a 28 kDa parasite protein gave a linear pattern in the cytoplasm of infected erythrocytes by IFA. Immunoelectron microscopy clearly revealed that the target antigen of these MAbs was located along the cytoplasmic clefts of infected erythrocytes. Immunoreactivity was also observed in association with vesicles scattered in the erythrocyte cytoplasm and vesicles of the CVC.
A double-labelling technique was applied to localize the 28 kDa and 95 kDa antigens in the same erythrocyte. The small gold particles identified the 95 kDa antigenic sites, while the large gold particles identified the 28 kDa antigenic sites. Immunoelectron microscopy demonstrated that small gold particles were associated only with vesicles and most of the large gold particles were seen in clefts. Some large particles, however, were found in association with vesicles together with small particles. Thus, double-labelling confirmed that the vesicles contained predominantly the 95 kDa antigen and some of the 28 kDa antigen, whereas clefts were associated only with the 28 kDa antigen.

The presence of *P. vivax* antigens in clefts and CVC indicates that these structures are related to trafficking of *P. vivax* antigen from the parasites to the erythrocyte surface membrane. Some *P. vivax* proteins, at least the 28 kDa protein, are transported from the parasite to the parasitophorous vacuole and to the clefts. The proteins are then transported along clefts and transferred to vesicles. The proteins moved toward the erythrocyte surface and into the caveola space. The proteins are then released from the caveola extracellularly. The vesicles might be formed by budding from the tips of lamellate clefts, similar to Golgi vesicles that are pinched off from the Golgi stack. On the other hand, a 95 kDa protein is present only within the vesicles and not in the clefts. This could indicate that the vesicles containing a 95 kDa protein may originate directly from the parasitophorous vacuole membrane. These observations indicate that host cell changes induced by *P. vivax* are involved in trafficking of *P. vivax* antigens to the erythrocyte membrane.

**Conclusions**

Host cell alterations induced by *P. falciparum*, *P. brasilianum* and *P. vivax*-infections were described by electron microscopy and post-embedding immunoelectron microscopy. *Plasmodium falciparum* infection induces knobs, electron dense material and clefts in the erythrocyte. Clefts are involved in exporting *P. falciparum* antigen from the parasite to the erythrocyte membrane. *P. falciparum* antigen is present in knobs that adhere to endothelial cells, causing the blockage of capillaries. Pf155/RESA, one of *P. falciparum* antigens, appears to lyse the erythrocyte cytoplasm and to assist in the release of gametes from the erythrocyte.

*Plasmodium brasilianum* infection induces knobs, short and long clefts and electron dense material, which are engaged in trafficking of *P. brasilianum* protein from the parasite to the erythrocyte surface. *P. vivax* infection induces caveola-vesicle complexes and clefts in the erythrocyte. They are also involved in trafficking of *P. vivax* protein from the parasite to the erythrocyte membrane. Our studies, therefore, indicate that host cell changes occurring in various species of malarial parasites facilitate the transport of malaria antigens to the host cell membrane.

**References**


Membranes are in constant flux with their surroundings. They exchange molecules with other membranes and receive or give up additional molecules in tune with the cell's physiological requirements. Some membranes can suddenly grow larger or retract in area. To alter membranes, the cell sometimes replaces an existing membrane with relatively large areas of preformed membrane while the putative original membrane is being removed as small vesicles. Thus, the cell has the capacity to modify its membranes to fit immediate as well as ongoing requirements.

As a hypothesis, we are proceeding in our research on the assumption that a given membrane can be modified \textit{in situ} to provide for changing roles. The well-documented receptor clustering into coated pits, and the sorting out and return of the receptors to the plasma membrane following ligand release in some types of receptor-mediated endocytosis,\textsuperscript{1,2,3} involves modifications of this type, albeit on a more limited scale than we envision possible. To test for broader possibilities of this hypothesis, we are investigating the membranes of the system of the free-living protozoan \textit{Paramecium}. These membranes are ideally suited to such a study because of the numerous functions they must assume in the life of a vacuole and their well-documented highly plastic nature.

Phagosome-lysosome membranes perform a range of critical functions for the cell. (1) The phagosome membrane encloses entering food and particulate matter and maintains an unbroken hydrophobic barrier between these potentially harmful substances and the cell's cytosol. (2) The mature phagosome membrane encloses a highly acid environment during the process of prey killing and digestion and probably contains the mechanism for the acidification of the phagosome. (3) These membranes protect the cytosol from digestive enzymes. (4) These membranes facilitate the movement of the products of digestion into the cytosol subsequent to the selective entry of acid hydrolases into the phagosome. (5) Finally, the various membranes of the phagosome-lysosome system have the capacity to recognize other membranes in a time-dependent preprogrammed manner.

The intent of this short review will be to illustrate the way the fine structure of one membrane is modified in synchrony with the changing state of the space enclosed by the membrane and to explain how this modification is brought about. Figure 1 provides a guide to the structures and membranes discussed in this paper.

**Membranes Belonging to the Phagosome-Lysosome System**

- Characteristics of the Cytopharynx and Nascent Phagosome Membrane
- Evidence for \textit{in Situ} Membrane Modification
- Fates of the Different Pools of Membrane Incorporated into the Membrane of Phagosomes or Phagolysosomes
- Discussion and Conclusions
- References

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**Membranes Belonging to the Phagosome-Lysosome System**

We have recently been able to determine with some certainty the total range of membranous vesicles and structures in \textit{Paramecium} that can be considered part of the phagosome-lysosome system.\textsuperscript{4} Using the lectin wheat germ agglutinin (WGA), all membranes previously known to be related to this system were labelled. These included not only those membranes around the discoidal vesicles, digestive vacuoles of undetermined stage, Lysosomes (shown by Lüthe and Plattner to bind to WGA-gold on Lowicry 1 K4M sections), but also the acidosomes, the \textit{trans cisterna} of the Golgi stack, a reticulum of membranes answering the description of the trans-Golgi network and a continuum of vesicles ranging from 70 nm to 500 nm or more.\textsuperscript{4} Using WGA-gold on frozen-thin sections of cells, which had been serially pulse-fed with latex beads of a different size for each
pulse and with the pulses interspersed with appropriate chase times allowing the labelled vacuoles to reach predetermined stages, we have shown conclusively that all vacuole stages label with WGA. Because the labelling with WGA is completely inhibited by triacetychitotriose and because Paramecium seems to lack sialic acid,\textsuperscript{6} we conclude that these membranes may be distinguished from other membranes by having in common a glycolcalyx containing either b-(1-4) oligomers of N-acetyl-glucosamine (b-(1-4) GlcNAc) or a very high content of GlcNAc. No label was found on the plasma membrane or coated pits at the cell surface. However, the membranes of crystal-containing compartments were heavily labelled, suggesting that these structures are related to the phagosome-lysosome system.

Having determined the full range of membrane-bound compartments associated with the phagosome-lysosome system, we can now look at the characteristics of its subdivisions.

**Characteristics of the Cytopharynx and Nascent Phagosome Membrane**

Freeze-fracture images\textsuperscript{7} as well as deep-etch, rotary shadowing (unpublished observations) show the membrane of the cytopharynx to have a highly particulate E-fracture face unlike the plasma membrane with which it is continuous. Discoidal vesicles have an identical E-fracture face. These two membranes also bear a glycolcalyx of identical appearance and thickness (10 nm).\textsuperscript{8} In addition, their similarities to each other and dissimilarities from other membranes of the phagosome-lysosome system are shown by the existence of a monoclonal antibody\textsuperscript{9} that binds only to epitopes found on these two membranes.\textsuperscript{10} These similarities, along with the morphological evidence of discoidal vesicles that have fused with the cytopharynx\textsuperscript{11}, indicate that nascent phagosomes obtain their membrane from the pool of discoidal vesicles.

**Evidence for in Situ Membrane Modification**

Again, freeze-fracture evidence has shown that the membrane of the early phagosome (also termed DV-I stage) is very quickly and dramatically altered so that by 15 sec to 1 min after pinching off from the cytopharynx its membrane no longer contains the high number of intramembrane particles (IMPs) on its E-fracture face but now is almost entirely devoid of IMPs.\textsuperscript{7} These particles are not simply transferred to the P-fracture face because the IMP number on this face remains about the same. The phagosomes at this stage are termed DV-II. The glycolcalyx of the membrane of these DV-II phagosomes are also altered by being reduced to a slightly uneven 7 to 10 min in thickness.\textsuperscript{8}

This membrane is identical in freeze-fracture\textsuperscript{12} and glycolcalyx\textsuperscript{8} appearance to a set of fairly large vesicles that bind to the nascent phagosome and travel with the phagosome to the cell's posterior pole. At the pole the vesicles are seen to fuse with the phagosome.\textsuperscript{13} Studies with weak bases such as neutral red and acridine orange show that these vesicles are acidic and, furthermore, the phagosome becomes acidic after, but not before, these vesicles have fused with the phagosome.\textsuperscript{14} Prevention of fusion by the application of cytochalasin B inhibits phagosome acidification. Thus, we have termed these vesicles acidosomes.\textsuperscript{14} Acidosomes and acidified-phagosome (DV-II) membranes have equal concentrations of prominent particles on their P-fracture faces\textsuperscript{7,12} which is quite different from the P-fracture face of the DV-I.\textsuperscript{7} The relationship between DV-II and acidosomes is further demonstrated by the unique presence of epitopes for a second monoclonal antibody\textsuperscript{9} that reacts with no other DVs.\textsuperscript{10} These epitopes are also found in the crystal-containing compartments (unpublished observation). We have always been impressed by the fact that the contents of acidosomes resemble the contents of the extracellular space in freeze-fractured replicas. This resemblance is even more pronounced in deep-etched images (unpublished observation). In NH\textsubscript{4}Cl studies we see that these acidosomes are sometimes labelled with horseradish peroxidase within a very short time, as short as 1 sec, following exposure of the cells to this enzyme marker (unpublished observation). Yet we have so far been unable to determine the origin of these acidosomes. Using deep-etch techniques following rapid freezing of living cells, we have recently observed a previously unrecognized set of small vesicles aligned along the same microtubules at the cytopharynx to which discoidal vesicles are attached (unpublished observations). Though not yet studied in detail, their proximity to the site at which acidosomes bind to phagosomes suggests a possible role for these vesicles in the formation of acidosomes should they coalesce to form the larger vesicles.

Our evidence thus suggests that shortly after they are formed phagosomes undergo a radical membrane replacement\textsuperscript{12} during which the original membrane is removed by vesiculation and the membrane remaining in the DV-II is, for the most part, derived from that of the acidosomes. The timing of this change corresponds to the time when the phagosome becomes acid, beginning at about 1 min and reaching a maximum acidity at about 5 min into the digestive cycle.\textsuperscript{12}
At this time Lysosomes can approach and bind to the DV-II. Lysosome membranes have a moderate number of very prominent IMPs on their E-fracture faces and an extensive 30 nm thick glycocalyx lining their luminal surface. Lysosomes also bear epitopes not shared by discoidal vesicles, acidosomes or the phagosomes. However, the membranes of phagolysosomes (DV-III) resemble Lysosomes in IMP number and distribution, appearance of their glycocalyxes and in monoclonal antibody specificity. Thus, the vacuole membrane has undergone another substantial modification, which occurs concomitant with fusion of Lysosomes beginning 8 minutes into the cycle.

The DV-III now contains acid phosphatase activity for the first time. The pH then rises dramatically. By the time the pH has returned to 6 or 7, digestion is presumably completed and a final membrane modification begins. Portions of the planar membrane are remolded into long tubules of a diameter of 45 nm, which contain acid phosphatase activity. These tubules expand at their distal ends into vesicles that resemble Lysosomes in all ways, IMP appearance, glycocalyx appearance, acid phosphatase activity and the presence of epitopes for the lysosome-specific monoclonal antibody (unpublished observation).

Vacuoles become defecation competent, under the axenic growth conditions we routinely use, at around 20 min. However, defecation of the labelled vacuoles in a population of cells follows an exponential pattern. The rate of defecation depends to a marked extent on the rate of vacuole formation. The membranes of these spent vacuoles (DV-IV), which are now acid phosphatase-negative, have not been studied extensively but they are probably quantitatively rather than qualitatively different from membranes of the DV-III phagolysosomes.

**Fates of the Different Pools of Membrane Incorporated into the Membrane of Phagosomes or Phagolysosomes**

At this stage in our story, solid evidence becomes scarce. Discoidal vesicle membrane seems for the most part to be retrieved soon after phagosomes are formed. Only one or two phagosomes per cell react with monoclonal antibodies specific for the discoidal vesicle and nascent phagosome membranes. We presume this membrane can be recycled to the cytopharynx for new phagosome formation but we have no hard evidence for this. The second group of monoclonal antibodies that are specific for acidosomes also bind strongly to a few vacuoles near the cytopharynx, but these epitopes seem to be diluted out of the vacuole membrane with time. However, even spent DV-IV contain some epitopes for the latter group of monoclonal antibodies. Lysosome membrane is retrieved from the older DV-III. Whether this retrieval is in bulk or is composed of only certain components of the Lysosomes is not known. Thus the spent vacuole membrane may be composed of a mixture of phospholipids derived from the three vesicle populations and may contain an assortment of glycoproteins and proteins retained in the vacuole membrane that were not sorted out and retrieved at earlier stages. Spent vacuole membrane apparently does not bear epitopes for discoidal vesicles, only a low level of epitopes for acidosomes, and few, if any, epitopes for Lysosomes. Spent vacuole membrane like other vacuole membranes is, however, highly reactive for WGA.

Once the vacuole is defecated, the DV-IV membrane is retrieved as tubules and vesicles of different shapes. At least some of these vesicles become discoidal, attach to microtubular ribbons and move toward the cytopharynx. This portion of the spent vacuole membrane is recycled as discoidal vesicles. With our new evidence for another set of small spherical vesicles, we will need to see if these arise also from the cytoproct. Horseradish peroxidase is inactivated below pH 5, so we will need to use another marker to be certain we do not miss vesicles that might have a very acid lumen.

**Discussion and Conclusions**

Morphological and monoclonal antibody studies provide conclusive evidence for the extensive in situ modification of an existing membrane that follows the changing physiology of the space that it surrounds. These changes can be explained for the most part by the insertion and retrieval of vesicular membrane of different vesicle populations. Whether slower membrane changes can occur by intercalation of individual molecules of proteins and phospholipids is not known.

Membrane modifications such as those reported here can be very fast and can involve large amounts of membrane surface area. To carry out such a rapid change, the cell sequesters preformed membrane around the membrane to be modified. This sequestered membrane is poised to fuse with only a limited subset of the vacuole population of the cell. Specific recognition factors must be present on the cytosolic side of the membrane of this subset of vacuoles to provide binding sites for only one specific set of vesicles. The nature of these recognition factors is completely unknown. Nor is anything known about the triggers that cause the
vesicles to fuse.

The putative recognition factors presented on the cytosolic vacuole surface must be replaced with time since acidosomes and, later, Lysosomes bind to the same vacuole. This replacement of recognition factors may be brought about by the addition to the vacuole membrane of vesicular membrane bearing the required recognition sites. Yet the answer is probably not this simple. In the case of lysosome binding, recognition sites for Lysosomes could be added to the vacuole during acidosome fusion, but Lysosomes do not bind directly to vesicular acidosomes free in the cytoplasm. Other factors must be active in the proper presentation of the putative recognition factors in a form that can be recognized by the vesicles.

Those aspects of membrane traffic in the phagosome-lysosome system in Paramecium remaining to be worked out include the origins of acidosomes and the identification of all sources of discoidal vesicles, that is, do these vesicles have any origin than that from the membrane itself. The ultimate origin of the lysosome membrane appears to be from the trans-Golgi network and the Golgi stacks. This membrane may represent the source of new membrane entering the system. Of immediate concern is the determination of the amount of intermixing of membrane components from separate sources and the determination if the cell has the capacity to sort and retrieve, as a package, those components that comprise uniquely acidosome, discoidal vesicle and lysosome membrane components. These questions are being studied using both morphological and biochemical techniques.

References


Figure 1. Schematic drawings of membrane flow and modifications in the phagosome-lysosome system of Paramecium. A. View of Paramecium from the dorsal surface. The anterior end (A) is to the left and the right side (R) is at the top. The buccal cavity (BC) is in a mid-ventral location and is continuous with the cytopharynx (CYX), where nascent phagosomes (NDV) form. Ribbons of
microtubules fan out from the cytopharynx, directing discoidal vesicles (DCV) to this region. Acidosomes (AC) bind to the NDV as it forms and travel with the phagosome (DV-I) as it moves along the postoral bundles of microtubules (POM) toward the cell's posterior pole. Fusion of the acidosomes and retrieval of DV-I membrane results in a smaller and acidified DV-II which binds to lysosomes (LY). Fusion of the lysosomes with the DV-II leads to a DV-III or phagolysosome. After digestion, lysosome components are retrieved, mix with primary lysosomes (pLY), which bud from the Golgi stack (GS) and/or the trans-Golgi network (TGN), and eventually bind to another DV-II. The spent vacuole (DV-IV), from which lysosome membrane components and acid phosphatase are removed, passes to the cytoproct (CYT), where it is defecated. Spent vacuole membrane is retrieved as tubular vesicles, some of which become discoidal vesicles. B. Membrane especially on the E-fracture face (E) but also on the P-fracture face (P). Another modification occurs in the appearance of the glycocalyx (gly) lining the luminal side of vesicle and vacuole membranes. The cytosolic (cyto) side of the membranes has not been studied but must bear recognition factors. In addition, different populations of vesicles have different antigenic binding sites, which are indicated here as circles, squares and rectangles on the luminal sides of the membranes. The precise location of these antigenic sites is not known.
The Link between Clathrin-Coated Pits and Receptor Mediated Endocytosis

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Cells constantly sample their environment by internalizing portions of the extracellular fluid and carrying the fluid in membrane-bound vacuoles to various intracellular targets. The efficiency of this process is increased when receptors for extracellular molecules are located at sites of internalization, a process called receptor-mediated endocytosis. For nearly 24 years the clathrin-coated pit has been implicated as the major site of internalization for receptor-bound ligands. There are two components to coated pit function. First, the polygonal lattice of clathrin or the clathrin associated proteins control the shape of the membrane leading to the formation of a coated vesicle. Second, molecular elements in coated pits cause certain membrane receptors to cluster so that when a ligand binds it is rapidly internalized. The goal of current research efforts, therefore, is to understand at the molecular level how invagination and receptor clustering are achieved.

The evidence that coated pits mediate the internalization of receptor-bound molecules is almost entirely derived from microscopic studies. The first description of this specialized region of surface membrane provided a confusing picture of its possible function; some researchers even thought coated pits were an artifact of tissue preservation. The observation that the number of coated pits is increased in oocytes that are internalizing yolk proteins, as well as the finding that ferritin binding sites are located over coated pits of reticulocytes, were two findings that suggested a function for this region of membrane. Beginning with the discovery of the low-density lipoprotein (LDL) receptor in coated pits, there are now some 20-25 molecules or macromolecular complexes that have been found to enter cells by this route. The list will undoubtedly grow; however, in each case the identification of the route of entry will depend upon microscopic techniques.

While progress has been made in identifying the receptors that use this internalization mechanism, researchers have made rapid progress in isolating coated vesicles, identifying and characterizing coated vesicle proteins, and delineating the architecture of the polygonal coat. Although many of the coat proteins have been identified and it is now possible to use them to assemble coated vesicles as well as coated pits in vitro, we know nothing about how coated pits function.
Inhibitors of Coated Pit Function

Despite the widespread morphologic documentation that certain ligands enter cells by coated pits, it is possible that static electron microscopic images do not reveal the true pathway of internalization. For this reason, many investigators have searched for drugs or culture conditions that would affect the function of coated pits in some informative way. Whereas there is no drug or chemical that specifically interferes with coated pit function, there are at least three culture conditions or treatments that profoundly affect coated pit function: depletion of intracellular potassium, incubation of cells in a hypertonic medium and acidification of the cell cytoplasm. Each treatment affects coated pits in a different way, which gives clues about the role of the clathrin lattice in endocytosis.

Larkin et al., were the first to describe a method for inhibiting receptor mediated endocytosis. Using I-labelled low density lipoprotein (LDL) as an endocytic marker, they found that when the level of intracellular potassium was lowered in cultured human fibroblasts below 40% of normal, the internalization of LDL was inhibited. Moreover, under conditions of maximal inhibition, these cells had a reduced number of coated pits on the cell surface; greater than 80% of the coated pits were missing. Daukas and Zigmond reported that the receptor mediated internalization of chemotactic peptide was inhibited in cultured polymorphonuclear leukocytes that had been incubated in a hypertonic medium. Hypertonic medium affects the endocytosis of LDL as well as bulk phase markers. As judged by immunofluorescence and electron microscopic evaluation of the inner surface of rapid-freeze, deep-etched membranes, cells incubated in a hypertonic media have a markedly reduced number of coated pits. Finally, Sandvig et al., found that when intracellular pH was lowered below pH 6.0, there was a dramatic and specific inhibition of endocytosis through coated pits. Unlike potassium depletion and hypertonic treatment, cells that are treated in this manner have normal numbers of coated pits; however, these pits appear to be paralyzed and unable to internalize ligand.

These studies imply that when clathrin lattices are absent from the surface membrane, a cell is unable to internalize receptor-bound molecules. Moreover, they indicate that the clathrin lattice has some direct role to play in the endocytic event because in acidified cells the lattice is still present but apparently unable to function. The exact reason for the effects of these agents on coated pit structure and function are not known. Therefore, these studies have failed to identify the active components that account for receptor clustering and invagination.

More than likely, the development of additional inhibitory treatments will not have sufficient molecular resolution to elucidate how coated pits achieve their endocytic function in cells. For this reason, there has been widespread interest in the development of in vitro methods for studying endocytosis. Our laboratory has focused on developing a method for preparing isolated plasma membranes that are capable of reproducing part or all of the endocytic cycle.

Reconstitution of the Endocytic Cycle in Vitro

The endocytic cycle in intact cells has several components: (a) receptor clustering over coated pits; (b) invagination of the coated pit to form a coated vesicle; (c) removal of the coat from the vesicle to form an endosome; (d) the sorting of receptor from ligand in an early endosome compartment; (e) the return of the receptor in a transport vesicle to the cell surface; (f) the movement of the ligand to a specific intracellular target such as the lysosome; and (g) the formation of a new coated pit at the cell surface. Our goal has been to develop the methodology needed for studying each step in vitro.
To accomplish this goal, we reasoned that we needed large numbers of isolated plasma membranes attached to a solid substratum by the extracellular surface. In these membranes, the numerous coated pits would be available for experimental manipulation. The method we chose relied upon the ability of cells to attach to a poly-L-lysine coated surface. The bulk of the cell can easily be removed by gentle sonication, leaving behind the plasma membrane. Since the membranes can be prepared on a variety of different surfaces, it is possible to analyse coated pits using carbon platinum replicas, indirect immunofluorescence or radioimmunoassay. With appropriate monoclonal antibodies, clathrin as well as important receptor molecules such as the LDL receptor can be easily detected by both visual and quantitative techniques.

Thus far our main focus has been on using these membranes to study coated pit assembly. The endogenous coated pits can be removed by brief treatment with a high pH buffer. This leaves behind sites on the membrane that are capable of initiating the formation of coated pits when suitable coat proteins are available. Cytoplasm prepared from tissues or cells can serve as a source of clathrin and clathrin associated proteins. Some of the important conclusions from these studies include: (1) clathrin and clathrin associated proteins are recruited from the cytoplasm to the surface of the cells and form normal appearing coated pits; (2) the membranes contain a limited number of assembly sites; (3) assembly occurs equally well at 4 °C and 3 °C with a half time of assembly of approximately 5 minutes; (4) assembly seems not to require a source of ATP; (5) when assembly is carried out at 37 °C, after initial assembly there is a rapid disappearance of the clathrin from the membrane, and this disappearance is inhibited by the ATP-destroying enzyme apyrase.

We have used this system to determine whether it is possible to assemble a coated pit from coat proteins that have been extracted from isolated coated vesicles. These coat proteins will form normal coated pits and the assembly reaction shares many of the properties described above.

This membrane preparation has also afforded us the opportunity to investigate whether the assembly sites have specific ultrastructural features as viewed in carbon platinum replicas of rapid-freeze, deep-etched membranes. The stripping procedure always leaves behind 20-30% of the clathrin that was originally on the membrane. The replicas reveal that this clathrin is in the form of incomplete polygons that are occasionally seen on the membrane. More importantly, these incomplete polygons are associated with well-defined particles that are arranged into clusters the size of a coated pit. These particles account for the fact that in stereo images, the flat lattices on unstripped membranes appear to be raised above the surface of the membrane; in other words, there's a definite molecular linkage between the clathrin lattice and the membrane. At least one component of the particles making up the assembly site most likely is the 100-50 Kd assembly complex identified by several laboratories as involved in coated vesicle assembly.

More recently, we have turned our attention to investigating whether the coated pits that are on these membranes are capable of rounding up and pinching off to form a vesicle. Although we have yet to prove definitively that endocytic vesicles can form from these membranes, we have found that the clathrin lattices will spontaneously round up when the temperature is shifted from 4 to 37 °C and that attendant with the rounding up process is the loss of clathrin from the membrane. The loss of clathrin is inhibited at pH below pH 6.0 and by treatment of the membranes with apyrase. Therefore, the behaviour of the clathrin coated pits in these isolated membranes is what one would predict based on the extensive morphologic studies carried out on a variety of cell systems.

**Future Directions**
To understand the molecular basis of coated pit function, it will be necessary to exploit an in vitro system such as the one we have developed. There is much to learn about the assembly phase. For example, we would like to know the nature of the assembly sites and whether or not their activity depends upon the assembly protein complex. More importantly, we would like to identify the membrane determinants that specify where and when a coated pit will form.

We are also optimistic that we will be able to use this system to study the invagination phase of endocytosis. If we can recreate this event and find conditions that lead to endocytosis, then there are innumerable studies that will be possible, ranging from the use of recombinant clathrin to the study of mutations that effect endocytosis. Ultimately, we would like to understand coated pits as dynamic cellular structures.

References


Cysteine Endopeptidases and Their Inhibitors in Tissue Invasion

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I shall review the ability of two particular proteinases to degrade extracellular connective tissue matrix components. Such activity is an essential aspect of the invasion of the tissues of the mammalian organism either by tumours or by parasites. Other pathophysiological situations in which such degradation is important include the remodelling of tissue structures in development and wound healing. The enzymes I shall consider are two cysteine endopeptidases.

The terms "endopeptidase" and "proteinase" are completely synonymous; "proteinase" is more familiar to most people, but "endopeptidase" is more rational, and is the term that we should use in the future.¹ Biochemists divide endopeptidases into four classes on the basis of the chemical groups responsible for their catalytic activity: these are the serine, cysteine, aspartic and metallo-endopeptidases.² It happens that representatives of all four groups are involved to a greater or lesser extent in the degradation of extracellular matrix components. Much recent work has emphasized the role of metallo-endopeptidases such as collagenase and serine proteinases such as leukocyte elastase. These enzymes have neutral pH optima, which obviously suit them well to extracellular activity, but the cysteine and aspartic endopeptidases have their part to play also.

One of the cysteine endopeptidases that I shall deal with is one from the Lysosomes of human cells, cathepsin L, and another is from the parasitic protozoan, Entamoeba histolytica, that we have called histolysin. I shall also mention the endogenous inhibitors that control the activities of these enzymes in the human body.

Cathepsin L

Human cathepsin L was first purified from liver, by Dr Rob Mason, in our laboratory.³ The amino acid sequence of the N-terminal part of the molecule shows a close evolutionary relationship with papain.⁴

One of the first natural substrates of cathepsin L to be discovered was Type I collagen.⁵ Much more recently we have discovered that cathepsin L also degrades elastin.⁶ Perhaps still more relevant to the issue of tissue invasion is the finding that cathepsin L is active on basement membrane collagen.⁷ There is good reason to think that the ability to penetrate basement
membranes is an important property in invasion.

I have broached the subject of the activity of cathepsin L against extracellular proteins without considering the question of whether the enzyme appears outside cells. Although usually considered an intracellular enzyme, cathepsin has been shown to be secreted from cells under conditions in which the degradation of matrix components occurs. Cells particularly active in the secretion of this and other Lysosomal enzymes include macrophages and osteoclasts. These cells are also known to create pericellular regions of acidic pH that are well suited for the extracellular action of the enzymes. It is therefore our view that cathepsin L is one of the battery of enzymes that play a part in the remodelling of connective tissue elements in a variety of pathophysiological processes.

**Histolysin**

Because of our interest in the contribution of enzymes such as cathepsin L to the breakdown of connective tissue elements, we were interested to read about a cysteine proteinase of *Entamoeba histolytica* that was thought to act in the same way. *Entamoeba histolytica* is the organism that causes amoebic dysentry, but it does not always confine its activities to the intestinal lumen. Sometimes it causes ulceration of the intestinal wall, invades the circulation and metastasises to other organs such as the liver, where it is able to set up secondary foci or infection. The escape from the intestine involves the disruption of the tissue structure, or "histolysis" - hence the specific name of the organism. It has been believed for a long time that endopeptidases secreted by *E. histolytica* play an important part in this process, but there has been little agreement on the properties of these enzymes. We were therefore delighted when a Cuban scientist, Alfredo Luaces, was able to join us for a year to do some work on the cysteine proteinase of *E. histolytica*.

Luaces grew the trophozoites of the axenic HM 1 strain of *E. histolytica* in culture and found that both the organisms and the culture medium contained cysteine endopeptidase activity. The activity was greatest in the organisms, so these formed the source used for the purification of the enzyme. We were fortunate to discover a method for affinity chromatography of the enzyme that led to a virtual one-step purification. 8 There was not much of the purified enzyme to spare, but we got a single automated sequencing run on the N terminus. A high proportion of the residues that were identified were identical with the corresponding residues in papain and cathepsin L, showing that all three enzymes are closely related in evolution.

We used the pure enzyme to study its catalytic activity. Like cathepsin L, histolysin has maximal activity on several protein substrates at acidic pH, but it differs in being stable at neutral and alkaline pH values, which would facilitate its extracellular activity. We tested it against matrix components at neutral pH. Contrary to some previous reports, we did not find that the enzyme was active on native Type I collagen; it is very active on denatured collagen, but that is probably not of much physiological significance. More importantly, the enzyme was active on the Type IV collagen of glomerular basement membrane, which could well help the organism escape from the intestine.

Equally important for invasion could be the ability to dissociate cells, so disrupting tissue architecture. Human skin fibroblasts in culture were transferred into serum-free medium and treated with 0.5 m a/ml of histolysin overnight. The cells rounded up, but they remained viable; this was not a cytotoxic effect, as has been claimed previously, but only cell detachment. Presumably this is due to degradation of some of the "sticky" proteins that cells produce, such as fibronectin.

**Inhibitors of Cysteine Proteinases**
I have spoken about two endopeptidases that have the potential to break down tissue structure by action on extracellular components. Before we can come to any sound understanding of whether such processes are significant \textit{in vivo}, however, we need to understand the systems that exist to control them. For cysteine endopeptidases, we specifically need to know about the cysteine endopeptidase inhibitor systems.

The first recognized inhibitor of cysteine endopeptidases in the human body was $\mu$-macroglobulin. This remarkable protein inhibits endopeptidases of all four catalytic classes by physically trapping the enzyme molecule within its own molecule. It is a very large molecule with a molecular weight of about 725,000. It exists in the plasma and to some extent in extravascular fluids.$^9$

The second class of inhibitors relevant to cathepsin L and histolysin comprises the cystatins. About 1980 we followed up earlier reports of the existence of a cysteine proteinase inhibitor in chicken egg white by isolating the inhibitor by affinity chromatography. We named it "cystatin", because of the way it stopped the activities of cysteine proteinase. We then developed the same type of purification procedure to deal with the more difficult task of isolating similar inhibitors from human liver. As a result of work in several laboratories, we now have a large family, technically a superfamily, of related cystatins.$^{10,11}$ Cystatins present in the human body are powerful inhibitors of both cathepsin L and histolysin. They would be likely to prevent such enzymes from causing any large-scale destruction of the tissues, but might be overwhelmed in the immediate vicinity of cells actively synthesizing and secreting the enzymes. One of the cystatins, human cystatin A, is located specifically in polymorphonuclear leucocytes and epithelial cells, suggesting that it may play a protective role.$^{12}$

References


Weak Bases as Inhibitors of the Trypanocidal Activity of Human Serum

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Effects of Weak Bases on the Trypanolytic Action
Characteristics of the Trypanolytic Factor
Conclusions
References

Among the African trypanosomes, the *Trypanosoma brucei* group is subdivided into the three subspecies *T. b. brucei*, *T. b. rhodesiense* and *T. b. gambiense*. The latter two are the causative agents of human sleeping sickness. *Trypanosoma b. brucei* and *T. b. rhodesiense* are practically indistinguishable using either morphological or biochemical criteria. However, the two subspecies can be differentiated on the basis of their sensitivity to the lytic action of normal human serum (NHS).

The phenomenon of the trypanolytic action of NHS has been known for more than 80 years, when Laveran\(^1\) was able to cure mice temporarily infected with *T. b. brucei* by injecting small quantities of NHS. Many attempts were made to identify and characterize the serum factor, before Rifkin\(^2\) identified a high-density lipoprotein (HDL) fraction as containing the trypanocidal activity. She also proposed later a model whereby the factor leads, via its interaction with the trypansome surface, to an alteration of the cell membrane permeability and a subsequent damage of the cell by osmotic shock.\(^3\)

A few years ago we decided to study the mechanism of this cytotoxic action of NHS more closely. Encountering difficulties in the isolation of a trypanolytic HDL fraction, an alternative method of isolating the factor had to be designed. In parallel, we looked for ways to analyse the lytic mechanism in more detail. Assuming that the trypanolytic activity (TLA) might occur intracellularly, acidotropic agents were used and their modifying effects on the lysis process studied.

Effects of Weak Bases on the Trypanolytic Action

5 × 10\(^6\) freshly isolated bloodstream forms of either the human serum-sensitive *T. b. brucei* strain STIB 345-A or the human serum resistant stock *T. b. rhodesiense* STIB 704 BABC were resuspended in 1 ml of MEM with 40% horse serum or 10% horse serum and 30% NHS. The extent of lysis was determined by taking aliquots of the cell suspensions incubated at 37 °C and counting the motile cells in a haemocytometer every hour. The percentage of lysis was calculated by taking the initial cell number as 100%. Under these conditions 100% of *T. b. brucei* are lysed within 6 hours, whereas *T. b. rhodesiense* showed no signs of lysis. Supplementing the medium with different chloroquine concentrations in the range of 10-25 mM resulted in a complete inhibition of the lytic action. Higher concentrations were toxic for both *T. b. brucei* and *T. b. rhodesiense*. Similar effects were found with other weak bases (Table 1).
The concentrations of the weak bases needed to inhibit the trypanolytic activity were in similar ranges to the concentrations reported to affect a variety of different intracellular processes associated with acidic compartments.\textsuperscript{4,5}

To test whether a preconditioning of trypanosomes with the weak bases would render them more resistant to the Lytic activity of NHS, \textit{T. b. brucei} were preincubated for 3, 2 and 1 hours in media containing either 30\% horse serum or 30\% NHS, both supplemented with 10 mM chloroquine. As a control, trypanosomes were incubated only in media with 30\% horse serum alone. After the preincubation period, all trypanosomes were pelleted and resuspended in 30\% NHS without chloroquine. No significant inhibition of Lysis was observed in any of the samples. On the contrary, a slight enhancement of Lysis was observed with the cells preincubated in NHS and chloroquine for 2-3 hours. These data seem to indicate that the trypanolytic activity can be efficiently inhibited only when the weak bases are continuously present in the medium. To prove that the weak bases were taken up, \textit{T. b. brucei} and \textit{T. b. rhodesiense} were incubated in the presence of different concentrations of chloroquine for three hours, washed once in PBS and kept frozen until chloroquine was determined with a quantitative high-precision thin-layer chromatographic method.\textsuperscript{6} It could be shown that the trypanosomes accumulated chloroquine (approximately 50-100 fold accumulation), but no concentration dependent uptake was found in the range of 0.5 to 20 mM. This can probably be explained by the fact that after three hours an uptake plateau is reached. Chloroquine determinations at much earlier time points might well show a concentration dependence.

Attempts to cultivate \textit{T. b. brucei} in NHS for longer time periods in the presence of chloroquine on a \textit{Microtus montanus} feeder layer failed.\textsuperscript{7} All trypanosomes died. Chloroquine probably accumulated intracellularly to toxic levels, as has been shown by others.\textsuperscript{8} In contrast, it was possible to maintain \textit{T. b. brucei} in 20 mM ammonium chloride for more than four days in the presence of normal human serum. The cells did not show any sign of lysis.

**Characteristics of the Trypanolytic Factor**

The trypanolytic activity in more detail it was necessary to isolate the trypanolytic factor. A new method to isolate the trypanolytic factor not involving ultracentrifugal flotation was established (Figure 1) using affinity chromatography of whole human serum on Blue Sepharose, followed by two anion-exchange steps and a final purification on Superose.\textsuperscript{9}

The known characteristics of the factor are summarized in Table 2. The factor consists of a macromolecular complex with a molecular weight over 1,000,000. Under non-reducing conditions a single polypeptide is found, which is separated into 3 4 peptides under reducing conditions. The isolated active factor was shown to be different from typical high density lipoproteins.

**Conclusions**

The trypanolytic activity of human serum can be inhibited by coincubation of bloodstream forms of \textit{T. b. brucei} with weak bases. It is proposed that the Lytic activity is mediated via a receptor-ligand interaction leading to endocytosis and a perturbation of the normal intracellular processing of receptor-ligand complexes. The perturbation of the processing of the receptor-ligand complexes might be prevented by the uptake of weak bases. Electron microscopic data indicate that significant structural changes occur in the presence of normal human serum in the region between the flagellar pocket and the nucleus, where endosomes and Lysosomes and the Golgi complex are known to be localized. For a detailed analysis of the cellular biochemistry of serum-resistant and susceptible trypanosomes, it is now necessary to find out precisely to which class of proteins the isolated trypanolytic factor belongs.
References


Table 1. Optimal concentration ranges of different weak bases to inhibit the trypanolytic activity

<table>
<thead>
<tr>
<th>Compound</th>
<th>Inhibitory concentrations (m M)</th>
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<tr>
<td>Chloroquine</td>
<td>10-30</td>
</tr>
<tr>
<td>Amantadine</td>
<td>50-500</td>
</tr>
<tr>
<td>Tributylamine</td>
<td>1-10</td>
</tr>
<tr>
<td>Ammonium sulphate</td>
<td>3-10</td>
</tr>
<tr>
<td>Ammonium chloride</td>
<td>5-50</td>
</tr>
</tbody>
</table>

Table 2. Properties of the isolated trypanolytic factor from human serum

- a complex of high molecular mass (>1000 kd) determined using gel filtration
- a single molecule under non-reducing conditions
- the factor is separated into three to four peptides under reducing conditions
- the major peptide has a molecular mass of » 80 kd
- the factor is present in human serum at a concentration of » 50 mg/l
- the isolated factor is not identical with high-density lipoprotein

Figure 1. Isolation of the trypanocidal factor from human serum.

Normal human serum

Blue Sepharose (albumin depletion)

Q-Sepharose anion-exchange

Mono-Q anion exchange (FPLC)

Polishing on Superose 6 (FPLC)

ACTIVE TRYPANOCIDAL FACTOR
Trypanosomiasis: Host Susceptibility and Trypanosoma brucei Lipid Uptake

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References

Trypanosoma brucei brucei, a causative agent of trypanosomiasis in domestic livestock, and T. b. rhodesiense and T. b. gambiense, the causative agents of human sleeping sickness, are tsetse-transmitted flagellated protozoa that multiply extracellularly in infected mammalian hosts. Variation of the surface glycoprotein (VSG) of T. brucei organisms prevents most hosts from clearing the parasites and leads to chronic and often debilitating infections. The pathologic consequences of trypanosomiasis caused by T. brucei and other pathogenic African trypanosomes are manifold, including: anaemia, raised Ig levels, immune complex disease, progressive destruction of lymphoid organs and other tissues, reduced fertility, cachexia and neurologic disorders. The severity of these disease symptoms varies among hosts, which, by this definition, are referred to as more trypano-resistant or more trypano-susceptible.

Immunity to T. brucei organisms is VSG-specific and is mediated by antibodies that react with exposed-epitopes on parasite VSG. It has been observed in model studies that prolonged infection with African trypanosomes leads to a general inability to mount humoral immune responses to trypanosomes and third-party antigens. A widely held view arising from these observations is that the level of susceptibility of a given host to infections with African trypanosomes is likely to reflect the rapidity of development and severity of parasite-induced immunodepression. In this formulation of the host susceptibility problem, the interaction between trypanosomes and the humoral limb of the immune system assumes centre stage and the attention of the audience/investigator is focused on parasite products that drive or disengage the circuits underlying immune responses.

Recent studies provide reasonable grounds to doubt the above concept. An extensive analysis of plasma-cell responses has been performed in resistant mice, which mount serologically detectable VSG-specific antibody responses and cause clearance of the first T. brucei parasitaemic wave, and in susceptible mice, which do not. In both strains of mice plasma cells arose with the same kinetics, reached similar numbers in all lymphoid organs examined and synthesized and secreted similar amounts of antibodies of the same Ig classes, including antibodies specific for exposed VSG-epitopes on the infecting organisms.

It was shown that the 2.5- to 10-fold higher peak levels of parasitaemia reached in the infected susceptible, as compared to the resistant, mice could result in the removal by the trypanosomes of large amounts of antibody. Hence it was inferred that there was less antibody bound per trypanosome, leading to failure of the susceptible mice to clear parasites from the bloodstream. VSG-specific antibody absorbed by trypanosomes is endocytosed and degraded (D. Russo, P. Webster and S. Black, unpublished). Failure to clear trypanosomes from the
bloodstream leads to a prolonged parasitaemic wave, rapid destruction of lymphoid organ architecture and concomitant loss of ability to mount efficient humoral immune responses.

Accessory studies showed that the higher levels of parasitaemia reached in infected susceptible, as opposed to resistant, mice correlated with slower parasite differentiation to committed non-dividing trypanosomes in the bloodstream, an event that is probably controlled by antibody-independent host responses. The rate of parasite differentiation to committed non-dividing T. brucei in the bloodstream of infected mice can be accelerated by treatment of mice with Propionibacterium acnes. Biological mediators induced by P. acnes have no direct effect on the parasites in vitro. The mediators examined include IL-1, IL-2, TNF, INF, PGE1, PGE2, PGF2, mitogen-induced mixtures of mediators derived from T cells, B cells, macrophages and mixed populations of cells (early or late after stimulation), fibroblast growth factors, endothelial cell growth factors, nerve growth factors, platelet-derived growth factor, insulin and insulin-like growth factors, tumor-derived growth factors and tumor-promoting factors (S. Black and J. Newson, unpublished). The observations led to the idea that regulation of trypanosome multiplication and commitment to non-dividing forms might be mediated by secondary physiological effects of immune mediators that regulated the availability or nature of host-supplied growth nutrients/growth inhibitors.

Axenic culture systems were exploited to identify host-derived macromolecules required for the multiplication of T. brucei. Two different serodemes of T. brucei were adapted to grow under axenic culture conditions and the adapted parasites grew equally well in both in vivo and in vitro environments. No major biochemical adaptations were thus required to transit between the two environments and hence the in vivo and in vitro T. brucei growth nutrient requirements were similar. The trypanosomes multiplied under axenic culture conditions in medium supplemented with 10% foetal bovine serum (FBS). In contrast, lipoprotein-depleted-FBS (LPD-FBS; density 1.25 gm/ml) did not support parasite multiplication unless supplemented with FBS lipoproteins (density 1.21 gm/ml). High-density lipoproteins (HDL; density 1.06-1.21 gm/ml) and low-density lipoproteins (LDL; density 1.006-1.06 gm/ml), prepared by sequential flotation ultracentrifugation, were equally able to supplement LPD-FBS to support T. brucei multiplication. Chylomicrons (density 0.96 gm/ml) and very low-density lipoproteins (VLDL; density 0.96-1.006 gm/ml) were unable to support T. brucei multiplication. Removal of HDL or LDL-lipids by alcohol/ether extraction abrogated the ability of the lipoproteins to support T. brucei multiplication. Both HDL and LDL from a number of different species, including cattle, African buffalo, eland, rabbits and rats, were as able as FBS-HDL or FBS-LDL to support T. brucei multiplication. The observations fit well with the published requirement of bloodstream T. brucei for exogenous lipids and suggest the presence of a lipid scavenging mechanism suited to a parasite with a wide host range.

Foetal bovine serum (FBS), rabbit and rat HDL and LDL were labelled with $^{125}$I on the apolipoprotein content, or with $^3$H cholesterol, $^3$H cholesteryl linoleate or $^3$H dipalmitoyl phosphatidyl choline, or with combinations of $^{125}$I and $^3$H labels. It was shown that both culture-adapted T. brucei and T. brucei isolated from the blood of infected mice (including organisms that had not been culture-adapted) (V. Vandeweerd and S. Black, unpublished) took up lipoprotein-lipids without taking up or degrading apolipoproteins. Uptake of the lipoprotein-lipids occurred at 37 °C but not at 0 °C to 4 °C, was saturable and was several thousand times more efficient than uptake expected to occur by fluid endocytosis. The uptake process did not discriminate between HDL and LDL, was independent of exogenous divalent ions and was not influenced by exogenous weak bases (20 mM ammonium chloride, 20 mM chloroquine). The uptake mechanism was thus utterly different from receptor-mediated endocytosis of LDL as practised by mammalian cells.
Uptake by *T. brucei*, X63 mouse myeloma cells and normal mouse spleen cells of lipoprotein-associated \(^3\)H cholesterol occurred to a similar extent. It resulted from desorption of the \(^3\)H cholesterol from the lipoproteins and its diffusion into the plasma membranes of the target cells. In contrast, uptake of lipoprotein-associated \(^3\)H dipalmitoyl phosphatidyl choline and \(^3\)H cholesteryl linoleate differed markedly among the three cell types. *Trypanosoma brucei* obtained these lipids from both HDL and LDL. X63 obtained the lipids from LDL only and normal mouse spleen cells did not take up the lipids.\(^8\)

Uptake by *T. brucei* of lipoprotein-associated \(^3\)H dipalmitoyl phosphatidyl choline was inhibited by including LPD-FBS in the incubation mixture. The active ingredient in the LPD-FBS was likely to be albumin. LPD-FBS stimulated rather than inhibited the uptake by *T. brucei* of lipoprotein-associated \(^3\)H cholesterol linoleate. This observation suggests that lipoprotein-derived phospholipids and cholesterol esters might enter *T. brucei* by different processes and that the phospholipid \(^3\)H dipalmitoyl phosphatidyl choline) is free of the carrier lipoprotein prior to entry into *T. brucei*.

In contrast, bile acids and conjugated and unconjugated bile salts inhibited the uptake by culture-adapted and normal bloodstream *T. brucei* of lipoprotein-associated \(^3\)H cholesteryl linoleate but not lipoprotein-associated \(^3\)H dipalmitoyl phosphatidyl choline. Different cholesterol conversion products had differing efficiencies to inhibit *T. brucei* lipoprotein-cholesterol ester uptake. The observed order, derived from inhibition of HDL-associated \(^3\)H cholesterol linoleate uptake using culture-adapted *T. brucei* as: lithocholic acid > chenodeoxycholic acid > deoxycholic acid > cholic acid = glycochenodeoxycholic acid > taurochenodeoxycholic acid (V. Vandeweerd and S. Black, unpublished).

Two possibilities present themselves. The cholesterol conversion products might prevent the uptake by *T. brucei* of lipoprotein-associated cholesterol ester by inhibiting cleavage to cholesterol that can readily diffuse across the cell membrane. Alternatively, the cholesterol conversion products might compete with an interaction between lipoprotein-associated cholesterol ester and a *T. brucei* cholesterol ester binding molecule. To examine these possibilities, purified lipoproteins were labelled with \(^3\)H cholesterol ether (\(^3\)H cholesterol oleoyl ether) and incubated with culture-adapted *T. brucei* in the presence or absence of an inhibitory concentration of chenodeoxycholic acid. In the absence of the bile acid, the *T. brucei* took up \(^3\)H cholesterol ether by a process that was similar in all respects to uptake of \(^3\)H cholesterol ester, e.g., uptake occurred only at 37 °C, was saturable, was inhibited by unlabelled lipoproteins and was enhanced by LPD-FBS. Uptake of the cholesterol ether was inhibited by the bile acid (Vandeweerd and Black, unpublished). Because cholesterol esterases are unable to cleave the ether bond\(^11\) and because cholesterol ether cannot diffuse through cell membranes, we conclude that *T. brucei* have a cholesterol ester/ether binding molecule that is blocked by cholesterol conversion products.

Both chylomicrons and VLDL contain phospholipids and cholesterol ester yet do not support *T. brucei* multiplication and do not inhibit the capacity of LDL or HDL to support *T. brucei* multiplication in vitro.\(^8\). It therefore seems possible that size constraints prevent interactions between the largest lipoprotein molecules and the *T. brucei*. This conclusion leads to the idea that lipoprotein/*T. brucei* interactions occur in the *T. brucei* flagellar pocket, from which the larger lipoproteins might be excluded. Alternatively, cholesterol esters and phospholipids might be sequestered in chylomicrons and VLDL in such a way that they cannot interact with *T. brucei* organisms. As a preliminary step to distinguishing between these possibilities, it is of some importance to define the maximum size of molecules that can enter the *T. brucei* flagellar pocket.

Uptake by *T. brucei* of HDL-associated \(^3\)H cholesteryl linoleate is inhibited by including puromycin or cycloheximide (20 μg/ml medium) in the incubation mixture. In contrast, uptake
by *T. brucei* of HDL-associated $^3$H dipalmitoyl phosphatidyl choline is not inhibited by including puromycin or cycloheximide in the incubation mixture. We therefore consider it likely that uptake of the $^3$H cholesteryl linoleate is mediated by binding to a protein, whereas uptake of $^3$H dipalmitoyl phosphatidyl choline is not. Based on these observations and the ability of LPD-FBS (albumin) to prevent uptake by *T. brucei* of lipoprotein-associated $^3$H dipalmitoyl phosphatidyl choline, we speculate that the $^3$H phospholipid is released from the lipoprotein particle by a process that does not require protein synthesis by the parasite and thereafter diffuses into the parasite membrane. It is tempting to suggest that release of the lipoprotein-associated phospholipid occurs in the *T. brucei* flagellar pocket as a result of mechanical disruption of the lipoprotein particle. The uptake by *T. brucei* of lipoprotein-associated cholesterol occurs as a result of simple desorption; uptake of phospholipid possibly occurs by mechanically induced release and diffusion. Uptake by *T. brucei* of lipoprotein-associated cholesterol ester may therefore be the only component of the parasite lipid scavenging mechanism amenable to specific chemotherapeutic or immunological attack.

Concentrations of bile acids (5 to 15 mM), and conjugated and unconjugated bile salts (50 to 100 mM), which inhibit uptake by *T. brucei* of lipoprotein-associated cholesterol ester/ether in the absence of LPD-FBS, are close to toxic concentrations. Although the inclusion of LPD-FBS in the incubation mixture reduces the short-term toxicity of cholesterol conversion products, it does not completely abrogate their effects. Concentrations of bile acids (100 mM) can be chosen that prevent multiplication of *T. brucei* in long-term cultures supplemented with 10% FBS. Because conjugated bile salts and bile acids are found in normal plasma, it is an attractive idea that these molecules might have a role to play in protection against African trypanosomes. Clearly, quantitative data are required on the bile acid, and conjugated and unconjugated bile salt concentrations in the plasma and interstitial fluids of normal and infected trypano-susceptible and trypano-resistant hosts. Equally clearly, quantitative data are required on the sensitivity of different trypanosome clones, serodemes and species to the toxic effects of cholesterol conversion products in the presence or absence of blood.

We hope that the above overview will stimulate further studies on trypanosome nutrient uptake, on the host/trypanosome interface as manifested in the *T. brucei* flagellar pocket and on the mechanisms used by infected hosts to control parasite growth.

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Free Radicals and Protein Damage: Consequence for Protein Function and Catabolism, and in Cytolysis

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Free radicals are produced inevitably during cellular metabolism, such as by electron leakage from electron transport chains and redox enzymes. In addition, leukocytes, and perhaps some lymphocytes, can produce a triggered extracellular flux of free radicals by means of a specialized electron transport chain assembled on the cell surface and involving a low-potential cytochrome, cytochrome b₂₄₅.₁,₂ This triggered radical flux is probably important in defence against foreign organisms, and may also have a positive role in the normal function of the cells. The primary radical produced in biological systems is the superoxide radical, but this may undergo conversion into many other radicals, including the extremely reactive hydroxyl radical. Reactions with lipids and possibly proteins may also deposit relatively stable hydroperoxides, analogous to hydrogen peroxide (the dismutation product of superoxide radicals). All these peroxides are important in that they represent a further source of radical generation after cleavage reactions involving transition metals. Table 1 gives a crude summary of the nature and actions of the biologically important radicals occurring in normoxic conditions.

Studies on the mechanisms of action of such free radicals initially concentrated on lipids and DNA as targets. More recently it has become apparent that proteins are at least as important as targets. I will outline our studies on protein damage by radicals, and indicate some of its consequences for functional activity, proteolysis and cytolysis. I will start with the exterior of cells, move to intracellular proteins and then discuss proteins at the interface, the plasma membrane and their involvement in cytolysis.

Extracellular proteins are catabolized mainly intracellularly after endocytosis. However, limited extracellular fragmentation of proteins may be a necessary preliminary to such endocytosis in the case of polypeptides of the fixed extracellular matrices such as cartilage. These matrices present the substrates in a compact non-diffusible form, and this limitation has to be overcome before substantial degradation can occur. In addition, extracellular degradation of diffusible proteins by limited proteolysis can occur, though it is usually restricted by the large extracellular concentrations of proteinase-inhibitors. We have recently studied the influences of free radicals on the generation of fragments from cartilage proteoglycan and their influence
on the proteinase-proteinase inhibitor balance.

In addition we have indicated that significant protein fragmentation may result in diabetes from radicals generated by autoxidation of extracellular glucose.

Our earlier studies\(^4\) on free radical attack on intact discs of nasal cartilage showed that defined radical fluxes (induced by gamma-radiolysis) could release macromolecules (biosynthetically labelled by \(^{35}\)sulphate) from the discs. Molecular characterization of these products revealed that virtually intact chondroitin sulphate was the primary product and implied that the main cleavage reactions were taking place on the polypeptide core, releasing oligopeptides attached to chondroitin sulphate side chains, which were themselves almost undegraded unless gross radical fluxes were used. Significant release of intact glycosaminoglycan could be obtained with doses of radicals corresponding to those that can be produced by a few million macrophages in a few hours, in other words, corresponding to biologically plausible doses, especially in inflammation. The limited degradation products of free radical attack on proteoglycans were characterized in more detail recently using purified proteoglycan monomer and intact proteoglycans as substrates. The data (Naish-Byfield and Dean, unpublished) confirmed that selective attack on the polypeptide core is a major event. A clear contrast in the nature of products released by chemical elimination reactions (glycosaminoglycan alone) and by radical attack (glycosaminoglycan attached to oligopeptide) was demonstrated.

Such fragments of connective tissue may possibly be generated in inflammatory conditions by the action of radical-generating cells such as macrophages. In the extracellular fluid they might then be subject to enzymic proteolysis. It has been suggested frequently that free proteinases may occur widely in these inflammatory circumstances because extracellular inhibitors such as alpha-1-proteinase inhibitor (\(\alpha\) 1PI) are selectively inactivated by free radicals, leaving free proteinase activity. However, we (Dean, Nick and Schnebli, unpublished) found that free radicals generated from transition metals with hydrogen peroxide have roughly the same capacity to inactivate three proteinase inhibitors (\(\alpha\) 1PI, secretory leukocyte proteinase inhibitor and eglin) as they do a relevant target enzyme (neutrophil elastase) when all are used at concentrations appropriate to the \textit{in vivo} condition (around 5 M). Since many extracellular proteins probably have detectable quantities of transition metals loosely bound (e.g., copper on histidine residues) and since hydrogen peroxide is the dismutation product of the primary radical generated by triggered leukocytes, this radical generating system is probably relevant (Table 1). On the other hand, some peroxy radicals may be more selective in inactivating \(\alpha\) 1PI (by virtue of its active site methionine, which is very susceptible to oxidation) than the other inhibitors and enzymes. Whether appropriate peroxy radicals occur in the extracellular fluid at appropriate places and times is rather debatable. So the suggestions that emphysema and other conditions in which extracellular proteolytic activity comes to exceed extracellular proteinase inhibitors may result from free radical affront are therefore insecure, though it is clear that localized environments may contain active proteinases.

As another extracellular event that may mark protein for complete intracellular proteolysis, we have studied the action of autoxidizing sugars on some soluble proteins. Partly consequent on radical generation, proteins are glycosylated. We now report\(^5\) that protein fragmentation takes place, with bovine serum albumin as target protein, using glucose and glyceraldehyde autoxidizing for one to eight days. This may cause accelerated catabolism of some proteins in poorly controlled diabetics.

The general point has been raised indirectly above that radical damage to proteins may be important in causing inactivation of protein function. If such non-functional proteins are to be removed, then it may be expected that some feature of the radical modification will lead to recognition of the protein by cellular degradative machinery. In the case of the extracellular
released fragments, this recognition is probably based usually on the adsorptive endocytosis of the materials: this can result probably from the unfolding of the protein moiety and the relatively increased exposure of hydrophobic areas, though other more subtle changes may also be important.

In the case of intracellular proteins, radical-mediated damage to proteins may also be important in establishing basal rates of catabolism of intracellular proteins. The consequent residue modification, fragmentation with some associated new N-termini, and unfolding may all help to 'signal' that a damaged protein is more available for catabolism by a variety of routes. For instance, by cytoplasmic mechanisms involving ubiquitin conjugation or by lysosomal routes involving exposure of hydrophobic surfaces on the damaged proteins, which facilitate their uptake into the site of ultimate catabolism. We have demonstrated that several conditions of elevated radical flux lead to an increased rate of proteolysis of bulk long half-life proteins in cultured cells and of mitochondrially synthesized proteins in isolated mitochondria. A greater acceleration of intracellular protein degradation after radical attack can be observed in erythrocytes. The roles of the several routes for intracellular catabolism of radical modified proteins are not established yet, though the main relevant possibilities are summarized in Table 2.

A striking feature of studies of effects on radical fluxes on cell catabolism is that while low doses of radical flux may cause accelerated catabolism, which can be construed as a detoxifying protective antioxidant function, higher doses often simply lyse the cells. We have investigated this to some extent in relation to macrophage-mediated lysis of T. brucei. We noted an interesting difference in sensitivity to lysis by radicals generated from hydrogen peroxide between bloodstream and procyclic forms, which to some extent could be abolished by removal of the glycoprotein coat of the bloodstream forms. Surprisingly, this coat seemed to be conferring increased sensitivity to radical lysis; this again indicates the possible importance of protein damage (as opposed to damage to other target molecules). Table 3 summarizes some evidence that macrophages can lyse T. brucei by means of their triggered radical production: the process can be inhibited by antioxidant enzymes that remove hydrogen peroxide (i.e., by catalase, or catalase with superoxide dismutase, but not by superoxide dismutase alone). It is interesting that the addition of metal is not needed in this system: it is provided by the medium. The failure of trolox (an amphiphilic water soluble analogue of tocopherol, vitamin E, the main lipid soluble chain-breaking antioxidant) to prevent cytolysis indicates that lipid peroxidation may not be critical in its mechanism.

We are investigating more closely the mechanisms involved in cytolysis of nucleated human macrophage cell lines by radicals generated from hydrogen peroxide and transition metals. We use a simple medium consisting of Hanks' Balanced salts solution, so that the addition of metal is unnecessary. Our concern is to establish whether protein or lipid damage, or both, are crucial early events committing cells to Lysis during such radical attack. We demonstrated that in the case of our cell lines, an event predictive (during radical attack) of Lysis and occurring earlier than any of those presently in the literature, is membrane depolarization. This occurs within a few minutes, while depletion of ATP and GSH is rather slow, and, indeed, not very extensive. Lysis follows the membrane depolarization events with a lag of the order of 1 hour. We argued that changes in membrane potential could reflect either lipid or protein damage. Using a very sensitive fluorimetric assay of lipid damage, we were able to detect lipid damage (as production of hydroperoxides) over a short period comparable with that of depolarization. However, we could completely abolish this lipid peroxidation (by the addition of antioxidants such as butylated hydroxytoluene) without affecting depolarization or subsequent Lysis significantly. In this respect the cells seemed to behave rather like the trypanosomes mentioned above. Therefore, amongst possible cell membrane targets, proteins seem to be the most likely site of primary damage leading to Lysis. In agreement with the comments
above on functional inactivation of proteins, we have shown that the activity of certain ion pumping proteins in the cell membrane, notably the Na/K-AtPase, is depressed over an appropriate time scale. It seems that proteins may be critical targets in cytolysis by radicals.

Thus protein damage by radicals is critical in many biological processes. This damage may lead to functional inactivation, but usually the inactivated proteins are probably degraded, so that proteolysis forms a secondary defence. However, when the target proteins are critical for rapid homeostatic mechanisms (as in the case of transport proteins) or when the proteolytic defence and/or other antioxidant defences are overwhelmed, toxic events may ensue. These are probably important in many chronic pathologies such as artherosclerosis (where damage to LDL may be critical) and chronic inflammatory diseases (where connective tissue catabolism probably involves some radical-mediated damage). The toxic events may even be so disastrous as rapidly to lyse cells.

References


Table 1. Some important Free radicals and related molecules in oxygenated biological systems

<table>
<thead>
<tr>
<th>Radical</th>
<th>Source/site</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Superoxide radicals (O2)</td>
<td>Electron transport chains (intracellular and in plasma membranes of some leukocytes, such as macrophages)</td>
<td>Not a very reactive entity; gives rise to H₂O₂ by spontaneous and catalyzed dismutation</td>
</tr>
<tr>
<td>Peroxyl radicals (RO₂)</td>
<td>Lipids, sugars and proteins that may be relatively stable</td>
<td>Selective in reactivity; generate hydroperoxides</td>
</tr>
<tr>
<td>Alkoxyl radicals (RO)</td>
<td>Lipids, other macromolecules, reactions of and from peroxyl radicals</td>
<td>More reactive than peroxy radicals in many contexts</td>
</tr>
<tr>
<td>Hydroxyl radicals (OH)</td>
<td>From some reactions of quinones and from reaction of transition metals with H₂O₂ (the Fenton reaction)</td>
<td>Highly reactive; rather unselective in action</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>From dismutation of superoxide radicals and from some oxidase reactions</td>
<td>Together with transition metals, generates hydroxyl radicals; diffusible through membranes</td>
</tr>
<tr>
<td>Hydroperoxides</td>
<td>On lipids and proteins</td>
<td>Can be fragmented by transition metals</td>
</tr>
</tbody>
</table>
Table 2. Possible mechanism for enhanced protein degradation after radical damage

After limited radical attack

\[ \text{polypeptide-histidine-polypeptide} \]

\[ \text{polypeptide + aspartate-polypeptide} \]

\[ \text{(enzymatic addition of N-terminal basic amino acid)} \]

\[ \text{(lysine or arginine)-aspartate-polypeptide} \]

\[ \text{recognition by the ubiquitin-dependent proteolytic system (present in most cellular compartments)} \]

\[ \text{enzymatic-proteolysis} \]

After extensive radical attack

1) unfolding of proteins makes them:
   a) more susceptible to proteinases
   b) more hydrophobic, and therefore more likely to enter lysosomes from the cytoplasm, or endosomes (and hence later lysosomes) from the extracellular fluid

2) Radical attack fragments proteins, and hence in the case of fixed proteins of extracellular matrices makes them diffusible and more accessible for endocytosis and degradation by cells such as macrophages


Table 3. Lysis of bloodstream forms of *Trypanosoma brucei* by the macrophage oxidative burst

<table>
<thead>
<tr>
<th>Conditions</th>
<th>% specific lysis by macrophages</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Untriggered</td>
</tr>
<tr>
<td>Control</td>
<td>0.3 (0.3)</td>
</tr>
<tr>
<td>(a) + catalase (2000 U/ml)</td>
<td>0.0 (0.5)</td>
</tr>
<tr>
<td>(b) + superoxide dismutase (300 U/ml)</td>
<td>1.0 (0.9)</td>
</tr>
<tr>
<td>(c) + catalase and superoxide dismutase (as above)</td>
<td>0.2 (0.6)</td>
</tr>
<tr>
<td>trolox (1mM)</td>
<td>2.1(0.6)</td>
</tr>
</tbody>
</table>

Bloodstream trypanosomes (biosynthetically labelled in their protein with leucine) were co-cultured with macrophages (from 7 day infected mice) at a 10:1 macrophage:trypanosome cell ratio for 2 hours. The medium was M 199 plus 2.5% rat serum. Phorbol myristate acetate (PMA) was at 50 ng/ml. Results are means (with s.d. in brackets, for n = 3). From ref. 9.
The Cross-Reacting Determinant of the Variable Surface Glycoprotein of Metacyclic Trypanosoma congolense

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References

The International Laboratory for Research on Animal Diseases was set up to address the problems of important animal diseases that affect livestock production in Africa, with its initial targets being trypanosomiasis and East Coast fever. The major emphasis of ILRAD's programme is in immunological approaches to the control of these two diseases. However, in order for such an approach to be successful, it is necessary that a parasite antigen, or antigens, be found that are accessible to antibody attack in vivo. In the case of African trypanosomes, the major surface proteins, which are called variable surface glycoproteins (VSGs), elicit a substantial immune response. However, these parasites ensure the successful continuation of succeeding waves of parasitaemia by changing their surface VSGs in a process called antigenic variation.¹

Although the surface coats of the African trypanosomes undergo continuous waves of change during the course of an infection, the different VSGs all contain similar structures at the carboxy-terminal (C-terminal) end of the molecule. This moiety is believed to function as an anchor by which the VSG is attached to the surface membrane of the parasite.² This putative anchor has been shown to comprise a complex glycosylphosphatidylinositol (GPI) moiety. Also found in the C-terminal portion of the VSG is an immunologically cross-reactive determinant (CRD).³ Antibodies directed against the CRD epitopes react with VSGs from different species of African trypanosomes, as well as a variety of proteins from many lower and higher eukaryotes.² Galactose, glucosamine, inositol 1,2-cyclic phosphate, and 1,2-dimyristoylglycerol (1,2-DMG) have all been shown to influence the binding of anti-CRD antibodies.⁴,⁵ Interestingly, however, in the case of African trypanosomes, this cross-reactivity was initially believed to be observable only in VSGs released from the surface of the parasite* by the action of a GPI-specific phospholipase-C (GPI-PLC).⁵,⁶ GPI-PLC acts by cleaving the 1,2-DMG from the GPI moiety and results in the formation of sVSG (a soluble form VSG), which, when analysed by sodium dodecylsulphatepolyacrilamide electrophoresis (SDS-PAGE),⁶ has a slightly higher apparent molecular mass (Mr) than does the GPI-containing, membrane form VSG (mfVSG). The GPI-PLC activity is reported to be activated by thiol-reducing reagents and inhibited by a variety of metal ions and detergents.⁷,⁸,⁹,¹⁰ However, in some cases the effect of these reagents has not been distinguished from a possible effect of the reagent upon the substrate rather than the enzyme itself. Clearly, this is of significant importance in the understanding of the process involved in the exposure of the CRD and release of the VSG from its membrane anchor. We have studied this using bloodstream and procyclic forms of several species of African trypanosomes and, in more detail, bloodstream forms and metacyclic forms of T. congolense. (The metacyclic forms are the infective forms of the parasite that are transmitted by the tsetse fly when it takes a blood meal from a
mammalian host.) Like the bloodstream forms, the metacyclic forms have a surface coat that undergoes antigenic variation. The procyclic forms are culture-adapted forms that resemble the parasites that are found in the midgut of the tsetse fly; they have no surface coat and show no binding of anti-CRD antibodies.

(* Strictly speaking this may not be the case since, by using immunoelectronmicroscopy, anti-CRD reactivity can be observed in fixed parasites. The reactivity is observed both on the surface of the parasite and in internal organelles such as the endocytotic network and trans-Golgi apparatus. However, because the anti-CRD antibodies do not recognize the CRD epitope in healthy, living trypanosomes, the CRD is unlikely to be useful as a potential antigen for vaccination purposes.)

Bloodstream forms of T. congolense, when analysed by SDS-PAGE and western blotting techniques using affinity purified anti-CRD reactive antibody (IgG), gave a single reactive band with an Mr of approximately 55,000. In contrast, metacyclic forms of the parasites gave several more or less discrete bands in the Mr range between 49,000 and 57,000. In the latter case we were surprised to observe several phenomena that did not accord with the dogma of increased CRD exposure (i.e., increased anti-CRD antibody binding to VSG) with the mfVSG to sVSG transition.

We observed that the metacyclic forms of T. congolense have, as expected, a CRD epitope on their VSGs. However, the 1,2-DMG does not need to be removed from these VSGs in order to cause increased binding of the anti-CRD antibodies to VSG. Furthermore, the exposure of the CRD is exquisitely sensitive to the method of sample preparation. For example, in contrast to reduced samples, sonicated samples that were not reduced with dithiothreitol showed no reactivity with the anti-CRD antibody. A variety of metal ions or detergents were tested for their effects on the exposure of the CRD. The results varied according to the reagent added and whether or not the reagent was added before or after sonication. Different results were also obtained when samples were prepared by the freezing and thawing of hypotonic lysates rather than by sonication.

While our results do not necessarily negate previous conclusions regarding the relationship between the exposure of CRD and the removal of 1,2-DMG, they clearly show that the exposure of CRD does not necessarily mean that the 1,2-DMG has been removed, as has been suggested by some workers. Indeed, these workers suggested that the exposure of CRD could be used as an assay for the release of the myristate anchor by the GPI-PLC. Furthermore, our results show that the pathway to the exposure of the CRD is clearly a complex one that can be halted at various intermediate steps by appropriate handling of the samples and that the reduction of disulphide bonds plays an important and, perhaps, crucial role in these events. An unravelling of these "unfolding" steps may help us to elucidate the various steps involved in the synthesis, packaging, transport and breakdown of the molecules during the intracellular processing of VSG.

References


Control of Intracellular Digestion in Paramecium

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Food or digestive vacuoles (DV) in Paramecium have been the subject of investigations for more than a century. Because of their large size and rapid formation, these DVs can be readily studied using light microscopic techniques. In fact, phagosomal acidification was first observed in this cell type as early as 1893. However, not until the last ten to fifteen years have we come to appreciate the true complexity of the phagosome-lysosome system in this cell. Notwithstanding these complexities, there are major advantages of using this cell as a model to study the control of intracellular digestion. In Paramecium the duration of the digestive cycle is relatively short, and the digestive processes are sufficiently synchronous and separated in time to permit perturbation of individual processes in situ and dissection of these processes via a pulse-chase protocol. Thus, by following a number of parameters such as vacuolar pH, vacuole size, acid phosphatase activity and thin-section and freeze-fracture morphology we have been able to distinguish at least four digestive processes that occur during a digestive cycle. These four processes can each be subdivided into at least four steps as outlined in Table 1.

These processes are facilitated in the vacuoles by the sequential addition and/or retrieval of at least three separate morphologically and functionally distinct pools of membrane-bound vesicles. This results in the formation of four morphologically distinct stages of the vacuole, DV-I to DV-IV, with each stage having its own characteristics. The discoidal vesicles provide membranes for vacuole formation. The acidosomes are responsible for DV acidification, which kills the ingested microorganisms, denaturing their proteins and preparing the vacuoles for lysosomal fusion. The lysosomes contain a complement of acid hydrolases capable of degrading most of the ingested macromolecules. The degraded products are transported from the vacuole to the cytosol to be used for the cell's growth. Finally, following defecation of the undigestible vacuolar contents from the DV-IV at the cytoproct, the spent DV-IV membranes are retrieved and the resultant vesicles are transported along microtubular ribbons back to the oral region where they are reused in forming new DVs.

Having obtained a relatively complete picture of the number of processes and steps involved in the digestive cycle, we have now turned our attention to the study of the regulation of these processes, in particular to whether these processes have to take place in a sequential order and how each step or process affects subsequent ones. We used (1) a pulse-chase protocol together with exposing these labelled cells before the onset of each of actin filaments, (2) ionophores that dissipate pH gradients, (3) weak bases that elevate vacuolar and vesicular
ph and (4) trifluoperazine, a calmodulin antagonist. These cells were then analysed using acid phosphatase cytochemistry, monoclonal antibodies specific for different membrane pools, and lysosome morphometry, as well as the acidification and degradation assays developed for these purposes. Thus, while the above perturbants may exert additional inhibitory effects on cellular processes not measured in this study, the totality of the experimental data appears to support the idea that the digestive processes behave as a chain reaction where each event exerts some control over the subsequent digestive steps. In this report we attempt to provide a brief summary of our findings on the regulatory mechanisms of the phagosome-lysosome system. The results are divided into three sections, each describing the effects of one of the first three digestive processes on the subsequent process or processes.

Results

DV formation

Vacuole formation is the first process in a digestive cycle and obviously is also the first, and perhaps the most crucial, regulatory process in a digestive cycle. When DV formation is blocked, all subsequent processes are eliminated. However, when DV formation and the subsequent processes are allowed to proceed normally, we find that the formation rate determines, to a large extent, the defecation rate. This conclusion is derived from studying the formation rate when cells were pulsed continuously with latex beads. During such a continuous pulse, the accumulation of labelled DVs could be divided into two periods: an initial period of linear but rapid increase in DV number followed by a second period after the mean number of labelled DVs/cell had reached a plateau that was maintained for the remainder of the pulse (Figure 1). The presence of this plateau indicates that either Paramecium is quickly saturated with DVs after feeding so that no new DVs are formed and no labelled DVs are ingested, or that the rates of formation and defecation are equal. To distinguish between these two possibilities, cells were pulsed with fluorescent beads for one hour and divided into two portions. One portion of the cells was kept in the initial pulse, which showed no net change in DV numbers for the remainder of the pulse, and the second portion was washed briefly to remove the fluorescent beads and fed again with a second label of nonfluorescent beads. In the latter group, the loss of the older fluorescent DVs was linear at a rate identical to their original formation rate during the beginning of the first pulse. The rate of DVs formation after exposure to the second label was also linear and rapid, but when exposed to the larger bead size used in this experiment, the DVs/cell value plateaued at a higher steady level. Since new DVs are continuously being formed while old DVs are continuously being defecated, to attain a steady level of labelled DVs, the defecation rate has to equal the DV formation rate. Not only are the rates of formation and defecation equal, but we also find that the defecation rate appears to be governed by the formation rate. This is confirmed by comparing the formation and defecation rates when cells were pulsed with decreasing bead concentrations. Under these conditions, the DV formation rates were lowered and lower plateau levels of labelled DVs were obtained. For each bead concentration, the defecation rate was reduced to the same extent as the formation rate (Figure 2). These findings suggest that (1) the formation process is the key step in regulating digestion and (2) the defecation rate is governed by the formation rate.

DV acidification effects of blocking acidosome-DV fusion on vacuolar acidifications as well as the lysosome fusion-digestion and defecation processes

As a nascent DV grows in size, acidosomes bind specifically to its surface. These acidosomes, which are located mostly in the cytopharynx area, are irregularly shaped electron translucent vesicles. They are acid phosphatase negative and have a distinctive freeze-fracture morphology and deep-etched appearance (unpublished observation). Microfilaments are found in regions where acidosomes bind to the nascent DV and to the very young DV.
These microfilaments apparently play an important role in the acidosome-DV fusion, since perturbation by cytochalasin B (CB) results in a block in acidosome DV fusion.\textsuperscript{7,9}

When acidosome-DV fusion was blocked by CB, DV acidification as well as the subsequent third\textsuperscript{10} and fourth processes of the digestive cycle became severely affected. Acidification of DVs, the pH was measured with indicator-dye-stained yeast cells, was almost abolished.\textsuperscript{7}

Lysosome-DV binding, as determined by measuring the number of bound lysosomes per mm of cross-sectioned DV-membrane profile (Figure 3), and lysosome-DV fusion, as determined by the appearance of phagolysosomal membrane antigens in the DV membrane (Figure 4) and acid phosphatase in the lumen, were greatly reduced (Figure 5). In our numerous pulse-chase studies we have rarely, if ever, observed lysosomes bound to the newly formed DVs in unperturbed cells, but numerous lysosomes accumulate around the normally acidified DV-II. This resulting lysosome layer bound to the DV-II often becomes highly conspicuous.\textsuperscript{7} When cells were exposed to 0.3 mM CB, lysosome binding to DVs was practically eliminated; only one 20-min-old DV in the treated cells had associated lysosomes; 1.4 lysosomes/mm of DV-membrane profile. The other eight DVs had none or just a few scattered lysosomes associated with their membranes\textsuperscript{10} (Figure 3).

To measure the lysosome-DV fusion rate, we used two monoclonal antibodies specific for the phagolysosomal (DV-III) membrane\textsuperscript{10,11} (Figure 4) jointly with acid phosphatase cytochemistry (Figure 5). In control cells chased after a 3-min pulse in latex beads, the DV-III membrane antigens were not expressed on young DVs. As these DVs aged, they showed increasing binding to these antibodies so that all 20-min-old DVs were positive. The lysosome-DV fusion rates for both control experiments were 5.1%/min as calculated from the linear portion of the curve (Figure 4). In CB-treated cells only 20% of the labelled DVs expressed the phagolysosomal membrane antigens by 20 min. although this had increased to 40% by 45 min. making the lysosome-DV fusion rate only 1.3%/min. This represented a 75% inhibition when compared with the control rate. This inhibition was readily reversible; the removal of CB by washing resulted in normal rates of fusion after a lag of about 15 min. When cells with 3-min-old DVs in which acidification was nearing completion but the lysosome fusion step had not yet begun were exposed to CB, CB did not inhibit the fusion rates but did reduce the maximal extent of fusion to 78% of that in the control cells (Figure 4).

Using acid phosphatase cytochemistry\textsuperscript{12,13} as a lysosome-DV fusion assay, results obtained from four separate experiments were all similar to those obtained with monoclonal antibodies (Figure 5). When acidosome-DV fusion was blocked by CB, less than 5% of the labelled 15-min-old DVs were acid phosphatase positive. With prolonged exposure to CB, only 15% of the labelled DVs became positive, giving a lysosome-DV fusion rate (0.65%/min) of less than 8% of the control rate. Again, the inhibitory effect of CB was readily reversible; 40% of the DVs became acid phosphatase positive 20 min after a wash, as compared with 50% in the control (Figure 5). Proteolysis of phagocytosed FITC-albumin in CB-treated cells was also reduced to a similar amount (unpublished results). As regards the fourth process, DVs in CB-labelled DVs in untreated cells were not ingested for at least 45 min. whereas labelled DVs in untreated cells could be defecated any time after 20 minutes.\textsuperscript{14}

These results demonstrate that when acidosome-DV fusion is blocked, all subsequent processes are essentially blocked: vacuoles will not be acidified and will not acquire the lysosomal membrane nor the lysosomal acid phosphatase. Finally, proteolysis is essentially prevented and vacuole defecation is greatly delayed.

**Effects of inhibition of vacuolar acidification on the lysosome fusion-digestion and defecation processes**
NH₄Cl has been shown to raise the pH of the endocytic compartments. We have used yeast labelled with fluorescein isothiocyanate to show that the vacuolar pH was elevated from 3 to about 5.8 in the presence of 30 mM NH₄Cl (unpublished). Though vacuolar acidification was inhibited, this concentration of NH₄Cl had no observable inhibitory effect on acidosome-DV fusion. The net effect of this pH elevation that we observed is an inhibition of lysosome-DV fusion. When measured by the Gomori method, lysosome-DV fusion was seen to be inhibited by 40% (Figure 6) and proteolysis of phagocytosed FIT-albumin was inhibited by 72% (Figure 7,) with about half of this latter inhibition being attributable to the effects of NH₄Cl on proteolysis itself independent of its effects on vacuolar acidification and lysosome-DV fusion. Under this treatment the defecation process was also slowed down (unpublished results).

**Lysosome fusion-digestion, lysosome-DV fusion**

The extent of lysosome-DV fusion will indicate the percentage of DVs converted to phagolysosomes, thus regulating the efficiency of proteolysis. However, it appears that once the acidosomes fuse with the DVs, lysosomes are able to recognize and bind to these DVs. Thus, when cells were exposed to either NH₄Cl, monensin or FCCP after the acidification process had commenced, little inhibition of lysosome-DV fusion per se was noted.

**Lysosomal proteolysis**

When cells were pulsed with undigestible materials such as latex beads in axenic medium, the duration of the processing period was 28 min and t½ for defecation was 37 min. However, when cells were pulsed with beads and digestible material such as albumin, vacuolar acidification and lysosome-DV fusion were normal but the retrieval of acid phosphatase was delayed (Figure 8), and the duration of the processing period was extended to 43 min and t½ for defecation was 60 min (Figure 9). These results show that the fourth process of defecation is influenced by the duration and efficiency of proteolysis, both of which are influenced by the phagocytosed materials contained within the DVs.

**Summary**

These results show that in *Paramecium* a chain of events takes place whereby each event exerts some control over the subsequent digestive processes. Thus, when DV formation is blocked, the acidification, lysosome fusion-digestion end defecation processes will not take place. When acidosome-DV fusion is blocked by CB, vacuolar acidification fails to occur. Under the last condition, lysosome-DV binding and fusion as well as proteolysis are inhibited by 90% and the DVs will not begin to be defecated for at least 50 min. whereas labelled DVs from untreated cells start to be defecated after 20 min. When the decrease in vacuolar pH is inhibited by NH₄Cl inhibiting acidosome-DV fusion, the secondary effect is a much lower reduction in the lysosome-DV fusion rate than when acidosome-DV fusion is prevented. As any reduction in the rate of lysosome-DV fusion will result in a slower rate of phagosomes being converted to phagolysosomes, proteolysis in these cells was found to be reduced. When the rate of proteolysis is reduced and its completion delayed, retrieval of lysosomal membrane and enzyme will be delayed, which will result in an extension of the digestive period and/or a slower rate of defecation. This results in defecation commencing at a later time. Thus, the overall effect is that the time required to complete a vacuole cycle is dependent on a number of factors operating in a sequential manner on the vacuole. When one of these factors is compromised, the resulting effects will be propagated along the chain of steps. This leads to an extended life of the vacuole as well as a decreased membrane recycling rate.
Acknowledgements

We thank Ms. Mari Moore for her data for Figures 8 and 9. This research was supported in part by grants from the National Science Foundation, and the MBRS, MARC and RCMI programs of the National Institutes of Health, USA.

References

17. FOK, A.K. and M.S. UENO. In preparation.

Table 1. The phagosome-lysosome system in Paramecium

<table>
<thead>
<tr>
<th>Four processes</th>
<th>DV age (min)</th>
<th>Membrane structure</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formation and release of DVs</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a. old DV membranes recycle back to cytopharynx</td>
<td>0-6</td>
<td>discoidal vesicle</td>
<td>7</td>
</tr>
<tr>
<td>b. particles captured by oral and somatic cilia</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c. discoidal vesicles fuse with cytopharynx</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>d. a newly formed DV is released (DV-I)</td>
<td></td>
<td></td>
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<tr>
<td>Acidification-condensation</td>
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<td></td>
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</tr>
<tr>
<td>Acidosomes bind to the forming DV</td>
<td>4-10</td>
<td>Acidosome 3</td>
<td></td>
</tr>
<tr>
<td>----------------------------------</td>
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<td></td>
</tr>
<tr>
<td>Acidosomes fuse with DV-I</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DV condenses, fission of membrane tubules</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Vacular pH drops from 7 to 3 (DV-II)</td>
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</table>

**Lysosome fusion-digestion**

<table>
<thead>
<tr>
<th>Lysosomes bind to acidified DV-II</th>
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<tbody>
<tr>
<td>Lysosomes fuse with DV-II, forming a DV-III</td>
<td>8-20</td>
<td>Lysosomes 3-7</td>
</tr>
<tr>
<td>Proteolysis and DV pH returns to 7</td>
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<tr>
<td>Lyso. membrane &amp; acid phosphatase retrieved</td>
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**Defecation**

<table>
<thead>
<tr>
<th>Lyso. membrane and enzyme retrieval completed</th>
<th>20</th>
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<tbody>
<tr>
<td>Digestion-competent DV-IV moves to cytoproct</td>
<td>&gt;20</td>
<td>?</td>
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<tr>
<td>DV and plasma membrane fuse at cytoproct</td>
<td></td>
<td></td>
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<tr>
<td>Retrieval of spent DV membrane</td>
<td></td>
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</tr>
</tbody>
</table>

Figure 1. Cells were pulsed continuously with fluorescent beads of 0.06 m m (●) or washed with axenic medium for 3 min after 60 min of pulse (top arrow). Immediately after the wash (bottom arrow), cells were pulsed with 0.3 m m non-fluorescent beads and were scored for both fluorescent (○) and non-fluorescent (▲) bead-labelled DVs.
Figure 2. Cells were pulsed with varying concentrations of fluorescent beads of 0.26 m m in diameter for two hours and were fixed at various times during the pulse. After washing, fluorescent DVs in *100 cells were scored for each time point. For cells pulsed with the lowest two concentrations of beads, lucifer yellow was added to aid in the visualization of the labelled DVs.

Figure 3. Binding of lysosomes to the DVs in DV-age- and acidification-dependent. Because lysosomes in Paramecium contain a distinctive glycocalyx in their luminal surface and characteristic paracrystalline inclusions, they can be recognized without acid phosphatase cytochemistry. Micrographs used to determine the number of lysosomes per m m length of cross-sectioned DV-membrane profile were randomly selected. In untreated cells, values shown by the closed circles represent the exact DV age, whereas the open circles represent the mean of DV age in cells that were pulsed for 3 min. Cells treated with CB (▲, 0.3 mM) were not chased to remove excess latex beads. Each value represents measurements obtained from 1 to 10 micrographs of various magnifications.
Figure 4. Effects of CB on the rate of lysosome-DV fusion. To mark the age of the DVs, cells were pulsed with nonfluorescent latex beads for 3 min and chased in fresh axenic medium. Cells were fixed in 4% formalin in phosphate-buffered saline and acetone permeabilized before being incubated in either of the two phagolysosome membrane-specific monoclonal antibodies (189D1G12 and 320E7, as represented by the open and closed symbols, respectively), followed by the FITC-labelled second antibody. For each time, labelled DVs in 100 cells were scored for positive fluorescence and an average value for the percentage of fluorescence-positive DVs/cell was thus obtained. CB (0.3 mM) was added to cells with 15-s-old (○, ○) or 3-min-old (△, △) vacuoles. To test for reversibility of the CB effects, aliquots of cells (exposed to CB when their DVs were 15-s-old) were washed (arrow) after 15 min of CB exposure (n, o). Control cells (▼, ▼) were exposed to the same amount of DMSO (0.2% v/v) as the experimental cells but not to CB.
Figure 5. Effects of CB on acid phosphatase activity in labelled vacuoles. Cells, pulsed with latex beads and India ink and chased in fresh axenic medium, were air dried on albuminized slides and fixed for acid phosphatase localization by light microscopy according to the hexazoized rosaniline method of Barka and Anderson.\textsuperscript{12} CB (0.3 mM) was added to cells after a 15 s prepulse with latex beads (●). Aliquots of cells exposed to CB when their DVs were 15 s old were washed after 15 min (arrow) of CB exposure (○). Control cells (▼) were exposed to the same amount of DMSO (0.2% v/v) as the experimental cells but not to CB. Each value represents the average of 4 experiments.
Figure 6. Effects of NH₄Cl on the rate of lysosome-DV fusion when vacuolar acidification was inhibited. Cells were pulsed with small fluorescent beads for 3 min and then chased. NH₄Cl (30 mM) was added to cells containing 0-min-old DVs (▲). Cells with 3-min-old DVs (○) were also exposed to this weak base for the purpose of differentiating their effects on lysosome-DV fusion independent of the DV acidification process, which was well along at this vacuole age. Vacuoles in control cells (●) were not exposed to NH₄Cl. Cells were fixed in 5% formalin in 0.05 M cacodylate buffer at pH 7.2, washed in acetate buffer at pH 5.0, and incubated in the Gomori medium¹³ at 37 °C for 10 min. For each time, 200 fluorescent DVs were each evaluated for acid phosphatase activity.
Figure 7. Effects of NH$_4$Cl (30 mM) on the rate of FITC-albumin proteolysis. With (●) or without (○) this weak base, cells were pulsed with a mixture of latex beads and FITC-albumin from 0 to 3 min and chased. Cells were also treated with NH$_4$Cl at 6 min (▲); at this age NH$_4$Cl at 30 mM has been shown to exert no inhibitory effect$^{10}$ on the lysosome-DV fusion rate. For each time, TCA-soluble fluorescence is expressed in arbitrary units/mg protein.
Figure 8. The rate of acid phosphatase retrieval depends on the contents of the vacuoles. Cells were pulsed with fluorescent latex beads with (▲) or without (△) albumin for 3 min and were chased in axenic medium. For each time, 75-225 fluorescent DVs were evaluated for this enzyme activity.
Figure 9. The duration of the processing period (which is defined as the period of time when the DVs are not defecation competent) and the rate of defecation are dependent on the contents of the vacuoles. Cells were pulsed with latex beads with (●) or without (○) albumin for 3 min and were chased in axenic medium. Labelled vacuoles were scored for the determination of the processing period and the rate of defecation as described previously.¹⁶
Intracellular proteins are continually being synthesized and degraded back to amino acids. One important function of intracellular proteolysis is to eliminate from the cell polypeptides with highly abnormal conformations as may result from mutations, biosynthetic errors and postsynthetic damage. In recent years much progress has been made in our understanding of how such abnormal proteins are recognized and selectively eliminated. Such studies have led to the discovery of new proteolytic systems with unanticipated properties, which are reviewed in this article.

One important feature of protein degradation in all cells and even within organelles, such as mitochondria or chloroplasts, is that this process requires metabolic energy. On thermodynamic grounds, peptide-bond hydrolysis should be a spontaneous process and not require ATP. Our laboratory has therefore focused on understanding this ATP requirement because it represented an important clue to discovering the responsible degradative system, and because it suggested the existence of novel biochemical mechanisms. This research led to the discovery of a new soluble proteolytic pathway in mammalian and bacterial cells, as well as in mitochondria, which requires ATP hydrolysis for function. Our knowledge of this pathway is most advanced in *Escherichia coli*, which were first shown to contain a new type of enzyme, an ATP-dependent protease. The best studied such enzyme is protease La from *E. coli*; however, as discussed below, similar enzymes appear to be widespread. For example, a similar ATP-dependent protease has been demonstrated in the matrix of mammalian mitochondria, and an ATP-requiring protease complex that acts independently of ubiquitin has recently been demonstrated in the cytosol of mammalian cells. Also, in the cytosol of eukaryotic cells, there exists a very large protease complex (1.5 kDa) that specifically degrades proteins conjugated to ubiquitin; it also requires ATP hydrolysis to function.

**Protease La**

In *E. coli* the energy requirement for protein breakdown results in large part from the involvement of the ATP-dependent protease La. This enzyme is encoded by the lon gene and has been shown to catalyze initial steps in the selective breakdown of proteins with highly abnormal conformations and of certain normal proteins that are also rapidly degraded.
Protease La is an ATPase as well as a protease, and these two functions are tightly coupled. In fact, for every peptide bond hydrolyzed in proteins, the enzyme consumes two ATP molecules. In other words, protease La consumes almost as much energy in cleaving peptide bonds as the cell uses to form them.

One important property of protease La is its precise regulation and its activation by protein substrates. The binding of a protein substrate to the protease leads to a marked activation of the enzyme's capacity to degrade peptide bonds, as shown with exogenous model peptides. This effect is seen only with protein substrates, such as denatured proteins, which interact with a regulatory region outside the active site and thereby cause allosteric activation. Native proteins are not hydrolyzed and do not activate the protease. This mechanism probably helps ensure that the protease does not exist in an active form in the cytosol, causing cell damage, but it becomes active only when it binds to a potential substrate (e.g., an unfolded polypeptide).

Substrates also influence the activity of this enzyme through a novel ATP-ADP exchange mechanism. The tetrameric enzyme binds up to four molecules of either ATP or ADP, but it has an even higher affinity for ADP, which is a potent inhibitor of proteolysis. Protein substrates, such as denatured albumin, both stimulate the binding of ATP analogs to the protease and also induce the release of the ADP molecules bound to the enzyme. By contrast, native proteins that are not degraded do not have these effects. Thus, in vivo, ADP molecules are normally bound to the protease and inhibit its function, until a potential substrate interacts with protease La. This step causes release of the bound ADP, promotes ATP binding and enhances proteolytic activity. These unusual properties presumably have evolved to prevent inappropriate or excessive degradation of normal cell constituents.

In the past year, the complete sequence of the lon gene, which encodes protease La, has been determined by the dideoxy method. The enzyme contains consensus sequences found in several other ATP-binding proteins. However, no two sequences homologous to the catalytic sites of any other proteases were found in this enzyme. Thus, protease La seems to represent the first of a new class of proteolytic enzymes.

**The Heat-Shock Response and Protein Breakdown**

The cellular content of protease La is also carefully regulated at the transcriptional level. Our studies, and those of Neidhardt, indicated that the lon gene is a heat-shock gene, i.e., one of the cellular genes that is induced at high temperatures and a variety of other stressful environments. We have shown induction of protease La in other conditions where cells generate large amounts of abnormal proteins, e.g., after incorporation of amino acid analogs or after expression of cloned foreign proteins in the bacteria. Under such conditions, the increased production of protease La seems to help prevent the accumulation of the abnormal proteins by enhancing the cell's capacity to degrade such polypeptides. Accordingly, the rates of protein breakdown and the level of protease La are reduced in *E. coli* mutants (*htpR* strains), which have a defect in the expression of heat-shock genes. Also, when wild-type cells are incubated at high temperatures (42 °C) or when they synthesize large amounts of incomplete or missense proteins at low temperatures, the cellular content of protease La increases 2 to 4 fold. Concomitantly, there is an induction of the other heat-shock genes in the bacteria. The promoter region of the lon gene contains a sequence homologous to that in promoter regions of other heat-shock proteins. In eukaryotic cells the generation of large amounts of abnormal proteins also appears to initiate the heat-shock response.

For example, microinjection of denatured proteins into frog oocytes elicits expression of heat-shock genes. Furthermore, ubiquitin, a critical component of the ATP-dependent degradative system in eukaryotic cells, is a major heat-shock protein.
Other ATP-Hydrolyzing Proteases

In addition to protease La, *E. coli* has been found to contain another ATP-Mg-dependent endoprotease named protease Ti. This enzyme (Mr = 340,000) is composed of two components, both of which are required for proteolysis, but have distinct functions. One component, A (subunit Mr = 80,000), is a labile ATPase that is stabilized by ATP. The other component, P (subunit Mr = 20,000), is a heat-shock polypeptide containing a latent proteolytic site that can be labelled with diisopropyl-3 fluorophosphate. These subunits show no proteolytic activity unless they are reconstituted. The ATPase activity of the reconstituted enzyme is activated 2 to 4 fold by protein substrates. Thus, protease Ti shares many unusual properties with protease La, such as coupled ATP and protein hydrolysis and protein-activated ATPase. However, these functions in Ti are associated with distinct subunits that modify each other's activities. Since these findings are very recent ones, it is still unknown whether similar enzymes exist in other cell types.

A very similar enzyme to protease La has been found in the mitochondria from rat liver. The mitochondrial matrix contains an ATP-dependent pathway capable of completely hydrolyzing abnormal organelle proteins to amino acids. Furthermore, the mitochondrial ATP-hydrolyzing endoprotease functions independently of ubiquitin, like protease La. This enzyme is very large (550 kDa) and degrades proteins in an ATP-dependent fashion, rather than resembling the main proteolytic system in the eukaryotic cytosol. The mitochondrial enzyme even has a similar specificity for peptides as does protease La, and its ATPase activity is activated by protein substrates.

The Proteolytic Complexes in Mammalian Cells

Eukaryotic cells contain a 650 kDa (19S) multifunctional complex with multiple endoproteolytic activities. This structure is composed of 9-12 subunits of 23-34 kDa and hydrolyzes basic, hydrophobic and acidic peptide substrates as well as proteins. It has been called by many names (e.g., the multicatalytic protease, macropain, LAMP), but we recently suggested the name "proteasome" to reflect its proteolytic activity and its identity with the cylindrical particle known as the "prosome" and present in the eukaryotic nucleus and cytosol. Prior studies had noted a small activation of this enzyme by ATP. Recently, using rapid chromatographic procedures and glycerol as a stabilizing agent, we isolated from skeletal muscle and liver a form of this enzyme that displays a large ATP stimulation. Hydrolysis of peptide substrates was stimulated up to 9 fold by ATP and of casein 4 to 6 fold. Neither ADP nor AMP had any effect, nor do nonhydrolyzable ATP analogs. This effect of ATP was very labile, and was lost with storage. The ATP-stimulated and independent "proteasomes" closely resemble each other (e.g., in apparent molecular weight, subunit composition and immunological reactivity). This enzyme does not require ubiquitin for activity, and thus it may be responsible for the ATP-stimulated hydrolysis of proteins that cannot be conjugated to ubiquitin (e.g., ones that lack free amino groups). Although its precise role in intracellular protein breakdown is still uncertain, this enzyme is clearly the major proteolytic activity in the cytosol of mammalian cells at neutral pH.

The elegant studies of A. Hershko, A. Ciechanover and co-workers have shown that the ATP-dependent pathway in mammalian cells requires the heat-stable polypeptide, ubiquitin. In this process, protein substrates undergo conjugation to ubiquitin by a multistep process requiring ATP. This modification marks them for rapid degradation. Our laboratory and that of Rechsteiner have identified a very large (1.5 kDa, 26S) enzyme complex within the cytosol that specifically hydrolyzes these conjugated proteins. The ubiquitin-conjugate-degrading enzyme (UCDEN) and the proteasome differ in many respects, including size, subunit composition and requirement for ATP. However, these enzymes seem to co-purify and to function in concert during ubiquitin-dependent proteolysis, and we have found that monoclonal
antibodies against the proteasome block the ubiquitin-dependent pathway. Although the structural relationships between these two large enzyme complexes remain to be elucidated, it seems most likely that the proteasome functions as a subunit of the larger (26S) conjugate-degrading complex.

Prospects

Appreciable progress has been made in our understanding of protein breakdown in bacteria and mammalian cells, but many important questions are still unresolved. Greater knowledge about protein turnover and the heat-shock response in parasitic organisms is highly desirable. Many similarities, and important differences, may be discovered through such studies of protozoa. For example, it has been shown that the gene for ubiquitin in trypanosomes is induced by heat shock, but it is organized in a very different fashion from that in mammals, since it contains up to 50 transcripts of ubiquitin in linear array. The significance of this interesting difference is unclear, probably because virtually nothing is known about protein breakdown, the role of ubiquitin and the heat-shock response in these microorganisms.

Selected Reading

ATP-Dependent Proteases in Bacteria and Mitochondria


Proteasome


Ubiquitin and the Ubiquitin-Conjugate-Degradating Enzyme


Heat-Shock and Proteolysis


Localization of a Variable Surface Glycoprotein Phosphatidylinositol-Specific Phospholipase-C in *Trypanosoma brucei brucei*

D.J. GRAB and Y. VERJEE

*International Laboratory for Research on Animal Diseases*
*P O Box 30709, Nairobi, Kenya*

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**Methods**

**Results and Discussion**

**References**

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African trypanosomes, the causative agent of "nagana" in livestock and sleeping sickness in man, are protozoan parasites that are cyclically transmitted by tsetse flies (*Glossina* spp.). They have a 12-15 nm thick coat of a variable surface glycoprotein (VSG) covering the entire surface of the organism.¹ The generation of a new population of trypanosomes with a different surface coat has been shown to be the result of a clone-specific change in the expression of a particular VSG gene.²,³ It is clear that the ability of the parasite to alter its VSG² has hampered the development of a conventional vaccine against trypanosomiasis. Therefore, a clearer understanding of the biochemistry of the VSG may suggest alternative targets for immunological intervention.

The VSGs from *T. brucei* are proteins known to be attached to the plasma membrane via a glycosyl-phosphatidylinositol (GPI) anchor.⁴ In addition, the presence of the GPI anchor is probably responsible for the immunological cross-reactivity observed between different VSGs.²,⁴,⁵ It has been postulated that the VSG is selectively released *in vivo* from the plasma membrane of the trypanosome as a result of the removal of the dimyristol-glycerol domain from the phosphatidylinositol via an endogenous phospholipase-C (PLC).⁶,⁷,⁸ The form containing the entire GPI anchor is commonly referred to as the membrane form of VSG (mfVSG) while the form lacking the dimyristolglycerol moiety is known as the soluble form of VSG (sVSG). Using subcellular fractions, we have that the VSG-specific PLC resided in the membrane of the flagellar pocket.⁹ However, we could not exclude the possibility that the mfVSG phospholipase-C activity could be in other membrane enclosed compartments: coated vesicles, endosomes, or other prelysosomal compartments. Internal localization of the mfVSG-PLC has also been postulated by others.⁴ The present report is an attempt to address this problem further.

**Methods**

*Trypanosoma brucei* clone MITat 1.52 was grown in lethally irradiated rats (600 rad) and the organisms isolated from the infected blood using isopycnic Percoll gradients followed by DEAE-cellulose chromatography supplemented with nucleosides.⁹ The cells were washed twice in ice cold SHKE (250 mM sucrose, 50 mM HEPES, 25 mM KCl, 1 mM EDTA, pH 7.4), and a membrane fraction prepared by the method of Grab et al.⁹ The fraction with the highest specific activity for mfVSG phospholipase-C (which banded on top of 14.4% Percoll; Fraction
V) was then applied onto a 12.5-42.5% SHKE gradient and centrifuged (4 °C) for 16 hours in an SW-41 rotor. The gradient was collected and the fractions analysed for total protein, mfVSG-PLC, and adenyl cyclase. In one experiment the cells were resuspended in RPMI-1640 medium (10⁸/ml) supplemented with 100 μm hypoxanthine and BSA (10 mg/ml) and incubated at 37 °C for 30 minutes before fractionation. The BSA content was determined by conventional liquid phase RIA using rabbit anti-BSA serum (Miles-Yeda, Ltd.) and ¹²⁵I-BSA.

**Results and Discussion**

Data from several experiments showed that the mfVSG-PLC activity resided in two membrane fractions with densities of 1.13 and 1.14 gm/ml respectively (Figure 1), with equally highly specific activity in each. However, most of the enzyme was in the 1.14 gm/ml peak. Adenyl cyclase, a putative flagellar pocket membrane marker, was found in high specific amounts in the 1.13 density fraction as well as in two broadly banding fractions with average densities of 1.11 (flagellar pocket membranes?) and 1.09 gm/ml, with the bulk of the total activity residing in the lighter fractions (Figure 2). To clarify whether any of the mfVSG-PLC containing fractions could be of intracellular origin, the trypanosomes in one experiment were preincubated with BSA to load the endocytic compartments of the parasite. The 1.13 density fraction contained the highest specific amount of BSA (Figure 3).

Although preliminary, the data suggest that the 1.13 density fraction may be of endosomal origin resulting from the fusion of a relatively higher density mfVSG-PLC containing fraction (d = 1.14) with a lighter membrane component (density less than 1.12) of possible flagellar pocket origin. The intracellular localization of the mfVSG-PLC is also suspected by Bülow et al. The data and interpretation, although attractive, are still far from complete. One should be able to ascertain this by incubating the trypanosomes under conditions were BSA or horseradish peroxidase is known to load only the flagellar pocket region and other internal organelles. In addition, other fractionation methods using other physical parameters are being exploited: e.g., free-flow electrophoresis.

**References**


Figure 1. Sucrose density gradient analysis of Fraction V. The relative specific activity (RSA) and relative total activity (RTA) for mfVSG phospholipase-C (mfVSG-PLC) in various fractions are shown.

Figure 2. Sucrose density gradient analysis of Fraction V. RSA and RTA for adenyl cyclase activity.
Figure 3. The relative specific content for BSA.
Understanding Human Lysosomal Diseases: A Review

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Our present knowledge about the biogenesis and function of lysosomes would never have been established without the presentation of a variety of lysosomal disorders by nature's diversity. Research about lysosomes provides an excellent example of cooperation between medical and biological sciences. Medicine cannot make any progress without basic science, but the advancement of basic science also depends on clinical observations and questions. It has been estimated that 1 in 100 children has a monogenic disorder. Metabolic diseases amount to only about 10 to 20% of the monogenic disorders and almost exclusively follow an autosomal-recessive or X-linked inheritance.

About 250 inherited metabolic diseases are known today and more than 30 have been identified as lysosomal diseases, which thereby represent the largest organelle-specific group. About 70 metabolic diseases affect the central nervous system. Of the 250 diseases, only about 20% have a treatment protocol. With regard to the frequency of the particular disorders, we can expect 1 to 2 children in 1,000 to suffer from an inborn error metabolism.

Lysosomes degrade almost any type of physiological macromolecule (Table 1). A storage disease will result either from a missing degradation step or from defective release of the degradation product.

Lysosomal storage diseases can be classified into three groups, shown in Table 2. The most frequent by far is the absence of an enzyme needed for degradation. This lack of enzyme activity can be caused by mutations leading to absence of the protein or to defective enzyme protein, or by defects of activators needed for the degradation of some sphingolipids, or by defects of protective stabilizing proteins or peptide sequences. The largest group of lysosomal disorders are the deficiencies of hydrolases involved in the degradation of heteroglycans, presenting as mucopolysaccharidoses, sphingolipidoses, glycoproteinoses and mucolipidosis I and IV.

The second type of lysosomal disease results from defective synthesis of the specific carbohydrate recognition marker as shown in mucolipidosis II and III. Research about these disorders has increased basic knowledge about the carbohydrate recognition marker.

The third group is likely to comprise transport defects through the lysosomal membrane. Examples in man are cystine storage in cystinosis and N-acetyleneuraminic acid storage in Salla disease.

There still remain diseases which from morphologic observations are likely caused by a lysosomal dysfunction but for which the biochemical defect has not been established yet. The different forms of the large group of ceroidlipofuscinoses and the Chediak-Higashi syndrome are among these diseases.

The importance of genetic lysosomal diseases in the understanding of lysosomal biogenesis and function is outlined in Table 3. Steps in the formation of lysosomal enzymes, some of them facultative (+/-), are listed in this table and examples for genetic defects are given.

How can inborn errors of metabolism been treated? Table 4 summarizes the therapeutic strategies that have been used to treat inborn errors of metabolism. Only three treatment methods are of potential use for lysosomal diseases. Removal of toxic material is the rational for the
treatment of cystinosis with cysteamine. When treatment is started early, the progress of the disease can be stopped. Cystinosis and Fabry's disease are diseases for which renal transplantation may be considered in the event of renal failure. Enzyme replacement might be a technique especially interesting for the treatment of lysosomal diseases, because the lysosomal enzymes exhibit a carbohydrate recognition marker for which receptors are expressed also on the cell surface pathway to the desired destination, the lysosome. However, practical realization of this concept is difficult, as summarized in Table 5.

Despite these difficulties, there have been many efforts to apply enzyme replacement for the treatment of human lysosomal diseases. All the earlier trials with discontinuous treatment have been unsatisfactory, because either the enzyme concentration was low, such as in plasma, or the side effects were not acceptable, such as after repeated buffy coat transfusions, or simply because of the technical problems to be overcome to gain the amount of enzyme needed for treatment. But the use of enzyme preparations for intravenous application is still being further developed. Molecular genetics allow the biosynthesis of large amounts of the missing enzymes. They can be modified to neoglycoproteins by adding carbohydrates, which can direct the enzymes to targeted organs. Another promising technique is to form more stable hydrolase-albumin complexes linked to antibodies that are directed to surface antigens of the targeted organ.

However, continuous enzyme replacement from transplanted organs seems to be more feasible. Bone marrow transplantation might be a viable treatment method for some lysosomal diseases. It has been shown that after bone marrow transplantation, a previously missing enzyme activity can be detected in organs such as the liver, spleen and kidneys. But for all diseases with CNS involvement, the blood-brain barrier seems to limit this approach. It is presently under discussion if this is completely true, because microglia cells, which comprise 5% of the brain cells, may be derived from bone marrow stem cells. If this proves correct, a very early bone marrow transplantation might even benefit those with lysosomal diseases with CNS involvement. However, in the diseases listed in Table 6, bone marrow transplantation already has been attempted as an experimental, sometimes desperate, trial. The presentation of data and the discussion of the results is very controversial and a definite recommendation cannot be given.

The strategy for the detection of a lysosomal storage disorder is outlined in Table 7. The cell fractionation technique we primarily use is free-flow electrophoresis. This technique enables us to purify lysosomes from almost any type of tissue culture cells, including lysosomal storage disorder cells with altered physical properties of lysosomes. It seems that free-flow electrophoresis is the method of choice for the isolation of lysosomes, especially from storage disease cells.

Many aspects of lysosomal diseases are still poorly understood (Table 8). There remain a great number of questions. One of these questions is: what makes the different phenotypic expressions of the same enzyme defect? For instance, the same enzyme, alpha-iduronidase, is defective in two different diseases, the mucopolysaccharidoses type Hurler and Scheie—both with similar skeletal symptoms, but the first with severe brain damage, the second without any. It is most likely that the explanation of such differences will come from molecular genetics. An important question is why and how a lysosomal storage process causes a disease; this question will be answered by biochemists and biologists. These are related problems. The first concerns the specificity of a disease caused by a certain storage product. What we need is more insight into the biochemical sequelae damaging cells and organs and leading to a disease. The other unsolved questions are what happens to the stored material: what is its final destination, how does it get there, does it affect endocytosis, is it undergoing exocytosis, does it affect the function of other cells or organelles? These questions can be answered only by continuous research and collaboration among biochemists, cell biologists, geneticists and clinicians.

**Table 1. Digestive function of lysosomes**
Table 2. Human lysosomal disorders

A: Defective enzyme activity
(missing or defective protein, defects of activator or protective protein)
- Mucopolysaccharidoses
- Sphingolipidoses
- Glycoproteinoses
- Mucolipidoses I and IV
- Glycogenosis type 2
- Acid lipase deficiency

B: Enzyme misplacement
- Mucolipidoses II and III

C: Transport defects
- Cystinosis
- Sialic acid storage diseases

D: Unknown
- Ceroidlipofuscinoses
- Chediak-Higashi syndrome

Table 3. Biogenesis of lysosomal enzymes
Table 4. Therapeutic approaches to inborn errors of metabolism

1. Dietary restriction of substrate
2. Replacement of missing product
3. Removal of toxic storage material
4. Activation of enzyme activity
5. Replacement of missing enzyme
6. Replacement of destroyed organ

Examples of inherited diseases:

Protein absent:
- Infantile glycogenosis II (Pompe)
- GM2-Gangliosidosis Tay-Sachs (Ashkenazi)

Protein inactive:
- GM2-Gangliosidosis Tay-Sachs (non-Ashkenazi)

Table 5. Enzyme replacement for the treatment of lysosomal disorders - methods and difficulties

<table>
<thead>
<tr>
<th>Methods</th>
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<tbody>
<tr>
<td>A: Discontinuous Therapy</td>
<td></td>
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<tr>
<td>1. Plasma infusion</td>
<td></td>
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<tr>
<td>2. Leucocyte transfusion (buffy coat)</td>
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<tr>
<td>3. Purified enzymes from urine, plasma, spleen and placenta</td>
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<tr>
<td>B: Continuous Therapy</td>
<td></td>
</tr>
<tr>
<td>1. Organ transplantation</td>
<td></td>
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<tr>
<td>2. Bone marrow transplantation</td>
<td></td>
</tr>
</tbody>
</table>
Difficulties

- Continuous supply of enzyme necessary
- Ligand properties of enzyme versus receptor specificity of target organ
- One enzyme form may not reach all tissues affected
- Blood-brain barrier?

Table 6. Bone marrow transplantation in human lysosomal diseases

<table>
<thead>
<tr>
<th>Mucopolysaccharidoses</th>
<th>Sphingolipidoses</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPS I (Hurler)*</td>
<td>Metachromatic Leukodystrophy*</td>
</tr>
<tr>
<td>MPS II (Hunter)</td>
<td>M. Niemann-Pick*</td>
</tr>
<tr>
<td>MPS IIIA, B (Sanfilippo)</td>
<td>M. Krabbe*</td>
</tr>
<tr>
<td>MPS IV (Morquio)</td>
<td>M. Gaucher*</td>
</tr>
<tr>
<td>MPS VI (Maroteaux-Lamy)*</td>
<td></td>
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</tbody>
</table>

*Animal model available.

Table 7. Strategy for the detection of lysosomal storage diseases

<table>
<thead>
<tr>
<th>Genetic transmission of a disease recognized (frequently associated with organomegally, +/- CNS damage)</th>
</tr>
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<tbody>
<tr>
<td>blood smear: lymphocyte vacuoles</td>
</tr>
<tr>
<td>tissue biopsy: intracellular storage vacuoles (e.g., liver, kidney, nerve tissue, bone marrow)</td>
</tr>
</tbody>
</table>

Analysis of storage tissue: Identification of lysosomes:
- Histological differentiation by staining procedures
- Isolation and chemical analysis of storage material
- Immunocytochemistry
- Cell fractionation with co-purification of lysosomes/storage material

Table 8. Open questions about lysosomal disorders

1. Different phenotypic expressions of the same defect (e.g., Huler/Scheie)

2. Relation of storage product to clinical expression
   a) Specificity of cell or organ alteration by a particular storage product (e.g., cystine, sialic acid)
   b) The fate of undigested material:
      - Is the endocytic pathway affected?
      - Is exocytosis involved?
      - Is the cooperation of lysosomes with other cell compartments disturbed?
The technique of introducing exogenous DNA into living cells, known as transfection, is a commonly used and very important approach in molecular biology. A number of methods have been developed to achieve this, but none of them can likely be used in vivo studies. In this paper we would like to introduce a pH-sensitive immunoliposome delivery system that can efficiently deliver such macromolecules in vivo in a target-specific manner.

Mechanism of delivery

Immunoliposomes are lipid vesicles coated with antibodies that have been attached with hydrophobic anchors. These surface antibodies direct the specific interaction of liposomes with antigen-expressing cells. Previous studies revealed that the bound vesicles are taken up by cells through a receptor-mediated endocytotic process. To prevent liposomes and their contents from being delivered to the lysosomes for degradation, pH-sensitive immunoliposomes are developed to enhance a prelysosomal discharge of the liposome contents into the cellular cytoplasm.

It was found for the first time in this laboratory that liposomes composed of unsaturated phosphatidylethanolamine and a weakly acidic amphiphile, such as fatty acid, become destabilized and fusion-competent when the pH of the medium is reduced to below 6.5. If these types of liposomes are endocytosed into endosomes that have a pH range of 5 to 6.5, they will release their contents into cell cytoplasm by fusing with the endosome membrane from within and/or rupturing the endosome membrane. The prelysosomal discharge of the liposome contents significantly enhances the cytoplasmic delivery efficiency of the liposome for anti-tumor drugs, toxin and DNA.

Delivery of plasmid DNA in vitro

In our model target system, monoclonal antibodies, anti-H2Kk, are used to direct liposomes to either mouse Ltk or RDM-4 cells that express the murine major histocompatibility antigen H-2Kk on the cell surface. Plasmid pPCTK-6A containing the herpes simplex virus thymidine kinase (tk) gene under control of a cAMP regulatory element, which is located at 5' upstream
of the rat phosphoenolpyruvate carboxykinase (PEPCK) gene - were encapsulated into:

1. pH-sensitive immunoliposomes composed of dioleoyl phosphatidylethanolamine P (DOPE):cholesterol: oleic acid (4:4:2), and


Both types of liposomes were able to deliver the plasmid to target cells as assayed by expression of the gene product (Table 1). But the amount of thymidine kinase activity in cells transfected by pH-insensitive immunoliposomes was eight times less than that transfected by pH-sensitive ones. Plasmid DNA entrapped in the pH-sensitive liposomes that bear no antibody also transform the cell phenotype less efficiently. Thus, both pH-sensitivity and liposomal antibody are important for efficient delivery. About 50% of the Ltk cells treated with the pH-sensitive immunoliposomes incorporated H-thymidine into the cellular DNA.

The long-term transformation efficiency of Ltk- cells by pH-sensitive immunoliposomes was also examined and compared with that by traditional calcium-phosphate precipitation method. We arbitrarily defined the "long-term transformation efficiency" as the proportion of treated cells that can undergo at least three cell divisions within 12 days in a selection medium. Therefore, a value of 5.5% was obtained for pH-sensitive immunoliposome method, whereas a much lower efficiency, 6 × 10⁻⁴, was obtained for the latter technique.

**Delivery of plasmid DNA in vivo**

To further demonstrate the utility of the pH-sensitive immunoliposome delivery system, a nude mouse model was used to show the target specificity of the DNA delivery. We entrapped into liposomes a plasmid, pBB 0.6, carrying *E. coli* chloramphenicolacety-1 transferase (CAT) gene under control of PEPCK promoter. The target RDM-4 lymphoma cells bearing H-2Kk antigen were grown as ascites tumor in Balb/c nude mice. A ³H-lipid marker was used to monitor the distribution of liposomes. Several treatments were performed, including:

1) Mice bearing RDM-4 lymphoma cells in peritoneal cavity and injected i.p. with pH-sensitive immunoliposomes containing the plasmid DNA

2) As in 1 except that liposomes are pH-insensitive composition

3) As in 1 except that liposomes are antibody-free, and

4) As in 1 except mice bearing no tumor cells

³H distribution in different organs revealed that without the presence of either tumor cells in the mice or antibody on the liposome, the majority of the liposomes were taken up by the spleen, regardless of the lipid composition of the liposomes. Moreover, liposomal antibody was important for targeting; twice the amount of antibody-bearing liposomes was bound to RDM-4 cells compared with the antibody-free liposomes.

The importance of liposomal antibody and pH-sensitivity for optimal delivery was further confirmed by expression of the CAT gene. CAT activity of RDM-4 cells from mice treated with pH-sensitive immunoliposomes was 6-fold higher than that with antibody-free liposomes. Up to 25-fold increase of CAT activity in the tumor cells was observed when mice were treated with pH-sensitive immunoliposomes instead of pH-sensitive immunoliposomes. Surprisingly, no significant amount of gene product was detected in other organs of the treated mice. The organ distribution of the CAT activity in mice treated with the pH-sensitive immunoliposomes containing the plasmid DNA is shown in Figure 1. It was evident that the expression of the
The pH-sensitive immunoliposomes are able to deliver macromolecules specifically to the cytoplasm of the target cell. The working mechanism of this system is summarized in Figure 2. The DNA delivery potential of the liposome has been demonstrated both in a tissue culture system and in an animal model. Future work will further develop the liposome system for cancer and viral chemotherapy, vaccine and gene therapy.

References


Table 1. Effect of liposome composition and targeting antibody on the thymidine kinase activity of Ltk cells

<table>
<thead>
<tr>
<th>Lipid composition</th>
<th>Antibody</th>
<th>Activity of tk (cpm/mg protein)(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DOPC:chol:OA(^b) (4:4:2)</td>
<td>+</td>
<td>9.7 ± 3.6</td>
</tr>
<tr>
<td>DOPE:chol:OA(^c) (4:4:2)</td>
<td>+</td>
<td>76.8 ± 28.7</td>
</tr>
<tr>
<td>DOPE:chol:OA(^c) (4:4:2)</td>
<td>-</td>
<td>11.7± 6.8</td>
</tr>
</tbody>
</table>

\(^a\) mean s.d. of 30 measurements.

\(^b\) pH-insensitive composition.

\(^c\) pH-sensitive composition.

Figure 1. CAT activity in ascites cells and organs of nude mice injected with pH-sensitive immunoliposome containing the plasmid pBB0.6. Mice were also injected with (open bars) or without (hatched bars) a mixture of 8-Br-cAMP and methyl-isobutyl-xanthine to turn on the expression of the CAT gene.

Figure 2. Schematic presentation of the interaction of immunoliposomes with the target cells. The liposomes bind to cell membrane by interaction between antigens ( ) and antibodies (*). First, liposomes appear in the endosomes (4) via endocytic pathway. Some of the endocytic liposomes may be transported into lysosomes which results in degradation of the liposomal DNA. Most of the pH-sensitive liposomes in the acidic endosomal compartments may fuse with endosomes that result in the delivery of DNA.
into cytoplasm. Alternatively, disruption of the endosomal membrane may also deliver the DNA into cytoplasm. The DNA delivered into cytoplasm may be transported into the nucleus by the "nuclear membrane binding protein(s)" or the "DNA-binding protein(s)". The exogenous DNA in the nucleus is eventually integrated into the chromosome that results in the expression of gene products.
Prior to secretion, secreted proteins are found in cytoplasmic secretory vesicles in eukaryotic cells. Secretion of protein almost universally involves fusion of the secretory vesicle to the plasma membrane. Although the mechanism of protein secretion appears to be the same in all eukaryotes, there is considerable variation in whether or not secretory vesicles accumulate in the cytoplasm, in how the secretion rate is regulated and where secretion occurs in the cell. Cells such as lymphocytes store very little assembled secretory protein in their cytoplasm, while cells such as exocrine cells and mammary epithelial cells (Table 1) store secreted proteins in large secretory vesicles that characteristically have electron dense cover.\textsuperscript{1,2} The rate of secretory vesicle fusion may be affected by no known stimulus, as in the case of lymphocytes or mammary epithelial cells, or it may be regulated by an intracellular second messenger, as is the case for endocrine, exocrine and neuronal cells, for example. Finally, the sites of secretory vesicle fusion may be restricted. As can be seen from Table 1, secretion can be apical, basolateral or non-polarized. Regulated secretion is found only in cells that have storage vesicles, consistent with a model in which regulated secretory cells store their newly synthesized secreted products until an appropriate extracellular signal triggers phasic release.

Newly synthesized membrane proteins reach the cell surface in transport vesicles. Because such transport vesicles have an internal volume, they will contain all types of newly synthesized secretory protein unless an exclusion mechanism operates. Conversely, storage secretory vesicle membranes will carry membrane proteins to the surface, unless there is a membrane protein exclusion mechanism. To test whether membrane proteins were excluded from secretory granules, we purified secretory granules from the mouse pituitary cell line, AtT-20, and showed that they lacked a newly synthesized membrane protein, gp70.\textsuperscript{3} We concluded that plasma membrane proteins were excluded from secretory granules and exited the cell by a different exocytotic pathway. This pathway we named the constitutive pathway, to distinguish it from the exocytotic pathway for release of storage granule contents, which had previously been called regulated secretion. The constitutive pathway was defined initially as the pathway that plasma membrane proteins take to the surface of cells. Externalization of membrane proteins was found to be stimulus-independent, whereas externalization by the regulated pathway was stimulus-dependent. Since some basal secretion can take place from the regulated pathway even in the absence of a stimulus, constitutive secretion is not the same as stimulus-independent secretion in our usage. Others equate the two, and so include in
constitutive secretion stimulus-independent release from secretory granules. We prefer to retain a definition of pathways that is based on partitioning newly synthesized proteins.

In the pituitary cell line, AtT-20, both membrane proteins and secreted proteins can be excluded from the secretory granule. The extracellular protein, laminin, appears to be one of the secreted proteins not in secretory granules. The simplest model that fits currently available data is that the secreted proteins that are excluded from the secretory granule exit the cell by the constitutive pathway taken by excluded membrane proteins. Direct proof of this model is lacking. There may be more than one pathway for constitutive secretion; fusion of immature granules could occur on the regulated pathway.

We have explored how different proteins are transported to the cell surface by transfecting DNA encoding secretory proteins into secretory cells. We discovered that proteins fell into two categories, those transported like ACTH, by the regulated pathway, and those transported like laminin, by the constitutive pathway. The data for two such proteins - trypsinogen and the kappa light chains of immunoglobulin - are given in Table 2 and compared with the secretion of ACTH, monitored by radio-immunoassay. There is a slowly turning-over pool for both ACTH and trypsinogen, which can be diminished by stimulation. In contrast, there is no pool with an unusually long half-time for the kappa chains, nor is the size of the pool affected by stimulation. The secretion rate of trypsinogen and ACTH is increased four to six times by a stimulus, while kappa secretion rates are unaffected.

We have attempted to look at what features of trypsinogen cause it to enter the regulated pathway using in vitro mutagenesis. Data on three mutant trypsinogens are compared with a wild type in Table 3. The sorting index for the wild type calculated in this experiment is 0.10. One of the mutants, TB08, has approximately the same sorting index, while two (TB09 and TB10) appear to be sorted more effectively than the wild type. Increases of sorting efficiency on mutagenesis have also been reported for defective insulin.

**Sorting of proteoglycans into secretory granules**

We have compared the sorting of a free oligosaccharide chain to protein sorting. Dense core secretory granules contain a sulphated proteoglycan of the chondroitin sulphate type. The sorting of free glycosaminoglycan (GAG) side chains, unattached to polypeptide chains, can be determined directly by labelling cells with 35S-sulphate in the presence of the GAG chain initiator, umbelliferyl xyloside. When we apply our conventional sorting assay to the partitioning of free GAG chains between the pathways, we get a surprising result (Figure 1), namely that the sorting of trypsinogen and GAG chains are indistinguishable. It is unlikely that a protein and a carbohydrate can be recognized by identical sorting machinery. We are considering the following alternative explanations. (1) The GAG chains are synthesized in the secretory vesicles after sorting has occurred. If so, the remarkable similarity between the sorting indices for proteins and GAG chains would then be fortuitous. (2) The sorting index represents relative volume flow from the Golgi region to the two pathways, and constitutively secreted proteins are excluded from the regulated pathway. (3) Proteins and GAG chains condense together into a dense core in immature granules prior to sorting and are sorted together.

In addition to GAG secretion, we have re-examined the sorting of an intravesicular chondroitin sulfate proteoglycan that we had characterized earlier. Measuring the sorting index of this proteoglycan by conventional techniques, we discovered that the sorting index was eight times higher than that of trypsinogen. Such apparently efficient sorting is strongly reminiscent of that of the hybrid protein created by deleting the C-peptide from proinsulin. There are at least two possible interpretations of these findings, proteoglycan could be very efficiently
sorted, perhaps, for example, by forming the condensed core to which the hormones adsorb. Alternatively, the proteoglycan that we measure could be generated largely or exclusively within the secretory granule. This would give a misleading high sorting index. To incorporate the data on the hybrid insulin molecule sorting into these models we would propose either that the hybrid molecule, perhaps because of its inability to generate correct disulfide bonding, condenses on a core matrix more readily. An alternative explanation is that the antigenicity of the mutant proinsulin is generated only in the milieu of the secretory granule.

**Conclusion**

While there is no doubt some proteins are sorted into secretory granules and some are not, the mechanism of sorting remains obscure. It certainly does not seem to be as simple as targeting lysosomal enzymes to the lysosome. Mutations in proteins can increase their efficiency of sorting into pathways. Increases in efficiency would be consistent with models of sorting in which proteins need to come out of solution and condense to a solid matrix prior to sorting, a model consistent with current data from electron microscopy. The probability of coming out of solution to form a matrix could readily be enhanced by mutation of the three-dimensional structure. Likewise, a core model could explain the co-sorting of free GAG chains and proteins. Proteins excluded from the regulated pathway would be those excluded from the forming core, although the accuracy of current data would also fit with lack of condensation, but no exclusion from the core. Even if matrix formation underlies protein sorting in secretory cells, the problem remains how selected membrane proteins, the integral membrane proteins of the secretory granule, are segregated with the dense protein cores.

**References**


**Table 1. Secretion patterns in eukaryotes**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Storage in secretory vesicles</th>
<th>Regulated</th>
<th>Polarity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exocrine</td>
<td>+</td>
<td>+</td>
<td>apical</td>
</tr>
</tbody>
</table>
Storage of secretory vesicles is positive when secretory vesicles usually with characteristic dense cores accumulate in the cytoplasm. Secretion is regulated when a known extracellular signal increases or decreases the rate of secretory vesicle fusion. Polarity of secretion is defined for epithelial cells. To what extent endocrine cells should be considered as polarized is not clear at this time.

Table 2. Comparison of secretion properties in AtT-20 cells

<table>
<thead>
<tr>
<th></th>
<th>ACTH¹</th>
<th>TRYPsinogen²</th>
<th>kappa²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Half-time of storage pool (h)</td>
<td>18.0 ± 4.0 (3)</td>
<td>14.8 ± 1.7 (4)</td>
<td>1.8 ± 0.5 (3)</td>
</tr>
<tr>
<td>Released on stimulation (%h)</td>
<td>12.3 ± 1.3 (3)</td>
<td>17.1 ± 1.0 (4)</td>
<td>-1.3 ± 2.0 (3)</td>
</tr>
<tr>
<td>Secretion rate (stimulated/rest)</td>
<td>5.6 ± 1.5 (3)</td>
<td>4.1 ± 1.5 (5)</td>
<td>0.9 ± 0.5 (3)</td>
</tr>
</tbody>
</table>

The releasable pool is considered to be the entire intracellular ACTH concentration measured by radioimmune assay, or the amount that remains in the cell extract after an overnight label with 35S-methionine, and a several-hour chase. The half-time is calculated from the rate of release from the pool per hour. The amount released on stimulation is the fraction of the pool released in one hour in the presence of a stimulus, less the fraction released in the absence of a stimulus. The ratio of the secretion rate in the presence of the stimulus, in this case, 8-Br-cAMP, to that in its absence is given. The figures in parentheses are the numbers of measurements.

¹ Measurements made by radioimmune assay.
² Measurements made after a 15-hr label with 35S-amino acids followed by a 3 - to 6-hr chase.

Table 3. Sorting efficiencies of trypsinogen mutants

<table>
<thead>
<tr>
<th>Trypsinogen</th>
<th>Wild type</th>
<th>TB08</th>
<th>TB09</th>
<th>TB10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sorting index</td>
<td>-</td>
<td>70 - 79</td>
<td>145 - 149</td>
<td>243 - 245</td>
</tr>
<tr>
<td>Region deleted</td>
<td>0.10</td>
<td>0.08</td>
<td>0.20</td>
<td>0.21</td>
</tr>
</tbody>
</table>

The sorting indices were measured exactly as described by Moore & Kelly (1985) and Burgess et al. (1987).

Figure 1. Comparison of chase kinetics for glycosaminoglycans and trypsinogen. The data for trypsinogen were generated exactly as described earlier (Burgess et al., 1985) in that cells were labelled with 35S-amino acids for 15 hr. The rate of release at the end of the labelling period was determined by immunoprecipitation of labelled trypsinogen. Similarly, the amount secreted during chase periods of 0 to 3 hr, 3 to 6 hr and 6 to 9 hr. Also included is the increment in release rate between 6 and 9 hr, when 5 mM 8-Br-cAMP was included in the chase medium. To label free GAG, the GAG chain initiator, umbelliferyl xyloside, was added to the medium in the presence of 35S-sulphate, as described previously (Burgess and Kelly, 1984). The secreted GAG chains, identified by their characteristic "staircase" appearance on polyacrylamide gels, were quantified by
elusion from the gels.
Proteolysis and endocytosis in *Trypanosoma brucei*

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Materials and Methods

Results

Discussion

References

A resurgence of interest in proteolytic enzymes has occurred in recent years as a consequence of the greater realization of the importance of proteases in the control of intracellular metabolic processes.\(^1\) Their involvement is now known to extend beyond simple protein digestion or the regulation of blood clotting and complement activation, and involves such complex tasks as hormonal processing, intracellular protein transport and the regulation of metabolic pathways. However, most work has concentrated on mammalian cells and only a small amount has been done on microorganisms. For example, our knowledge of the proteolytic enzymes of African trypanosomes is confined almost entirely to those found in the endosomal/lysosomal system, namely, their acidic thiol-dependent proteases.\(^2\) In contrast, little or nothing is known about the proteases that are involved in the subtle regulation of their metabolic processes. In part this may be due to the presence of very high levels of lysosomal cathepsin-like activity in the African trypanosomes, which tend to mask the presence of other highly specific enzymes. Consequently, there is a need to find ways of circumventing the blanketing effect of the trypanosomal lysosomal proteases.

In general, proteases are classified according to the mechanism of action of the enzymes. This is frequently ascertained by employing specific inhibitors known to react with particular amino acid residues within the enzymes. For example, trypsin and related enzymes are shown to be serine proteases by their reaction and inhibition with diisopropylfluorophosphate (DFP), a reagent that reacts almost exclusively with serine residues at the active sites of these enzymes. Here we describe some of our observations on the uptake and hydrolysis of fluorescently labelled materials by African trypanosomes as well as some effects of protease inhibitors on these organisms.

**Materials and Methods**

Analysis of the thiol-dependent proteolytic activity of African trypanosomes, and of the serum derived modulator of these proteases, was done by electrophoresis in fibrinogen containing sodium dodecysulphate polyacrylamide gels (Fbg-SDS-PAGE) as described previously.\(^2,4\) Detection of serine esterases was performed by incubating \(^3\)H DFP (Amersham, UK) with the respective material as described by Scheiner and Quigley.\(^5\) The uptake of 3 m \(^3\)H DFP by *Trypanosoma brucei*\(^6\) was performed in pH 8.0 phosphate buffered saline glucose (PSG). Uptake of rhodamine-lisamine-BSA (Molecular Probes Inc., Eugen, OR, USA) was performed on an SLM Aminco 8000 spectrofluorometer (excitation, 560 nm; emission, 590 nm) using
trypanosomes isolated and incubated (in PSG pH 7.4) in the presence of 50 m M hypoxanthine.7 The hydrolysis of Z-Phe-Arg-NHMec was followed specrofluorometrically (excitation, 380 nm; emission, 460 nm).

Results

Trypanosoma brucei incorporate 3H DFP into TCA precipitable material. The uptake is dose and time dependent and conforms to a first rate order equation (Figure 1). The chloromethylketones Ac-Ala-Ala-Ala-AlaCK, MeOSuc-ValCK, TPCK, or TLCK had a statistically insignificant effect on the total uptake of 3H DFP by either trypanosomes or trypanosome lysates. Analysis of the proteins labelled with 3H DFP, by SDS-PAGE and fluorography, showed a doublet at 71 kDa, minor bands at 52 and 39 kDa and a band at 27 kDa. Only minor differences were observed between the control and TLCK or TPCK treated samples. Curiously, the presence of TPCK slightly enhanced the labelling of the 39 kDa band. A doublet of Mr approximately 70 kDa was labelled in living trypanosomes, the lower of which was only weakly labelled in homogenates of the parasites.

Analysis by Fbg-SDS-PAGE clearly demonstrates the presence of thiol-dependent proteases in African trypanosomes, which are apparently localized within their endosomal/lysosomal system.2 We have observed and now isolated, a moiety from rat serum which has the capacity to bind to and either stabilize or activate this lysosomal proteolytic activity. The molecule has a similar Mr to serum albumin but, unlike serum albumin, it does not bind to amicon blue dye-A. It also has properties similar to kininogens (pl 4.8) and is found in commercial preparations of human high and low molecular weight kininogens, but again, antibody reactivity suggests distinct identities of the molecules.

Characterization of the activating molecule is under way, but it is also necessary to ascertain whether it is taken up by African trypanosomes. We are therefore studying endocytosis by these parasites. Preliminary studies have been performed using Z-Phe-Arg-NHMec and rhodamine-BSA. Providing the parasites are properly temperature equilibrated, they show an almost immediate steady state hydrolysis of Z-Phe-Arg-NHMec. (If they are not temperature equilibrated there is a lag phase of 1-3 min before a steady state is reached.) The hydrolysis rate of Z-Phe-Arg-NHMec shows a non-linear increase upon increasing the temperature of the reaction medium. The fluorescence of rhodamine-BSA in T. brucei is localized between the nucleus and the flagellar pocket and its uptake is time, dose and temperature dependent.

Discussion

African trypanosomes contain acid2,8,9,10,11,12 and alkaline2,7,10,13,14 endopeptidase activities. They may also contain cathepsin-D-like activity, although data to support this is weak at present12 (J. Lonsdale-Eccles and G. Mpimbaza, unpublished observations). With respect to the alkaline pH peptidolytic activity we have already shown that trypanosomes contain a DFP and TLCK susceptible activity.2 We wished to see if there were additional serine peptidase activities in the parasites and so tried the method of Scheiner and Quigley5 to explore this possibility. Although we were able to incorporate DFP into discrete proteins, incubation with a variety of chloromethylketones failed to prevent the incorporation of 3H DFP into these molecules. In view of the fact that we have already seen a DFP and TLCK susceptible activity in trypanosomes, it seems unlikely this observation may be the result of the specificity of the enzymes being too tightly defined for the chloromethylketones concerned. It seems more likely that the levels of TLCK and TPCK susceptible serine protease activity in the parasites may be lower than those of the enzymes detected in the SDS gels. Thus, the observed 3H labelled bands in Figure 2 are probably not proteases but rather serine esterases with, as yet, ill-
defined specificity. Pertinent to this is the observation that *T. brucei* contains at least two particle-bound phospholipases which are differentially inhibited by DFP.\(^\text{15}\) While the situation with respect to serine proteases is still unclear, the situation with respect to the thiol-dependent acid proteases, which we have termed trypanopains, is better defined. One such enzyme has been isolated and purified from *T. congoense*.\(^\text{11}\) A similar activity has been located in subcellular fractions containing lysosome-like organelles from *T. brucei*,\(^\text{2}\) and in equivalent fractions from *T. congoense* (J. Lonsdale-Eccles and D. Grab, unpublished observations). Their intralysosomal location would suggest that they play a role similar to that played by the corresponding mammalian intralysosomal activities although this has not yet been delineated. Nor is it clear what controls the activity of these enzymes. However, we have observed that plasma contains a moiety that can bind to and apparently activate or stabilize trypanopain activity when analysed by Fbg-SDS-PAGE.\(^\text{2,4}\) Curiously, preliminary experiments using Z-Phe-Arg-NHMec showed no apparent enhancement of activity with this moiety. This may mean that the binding of the serum moiety occurs at a regulatory site removed from the active centre of the trypanopain, thereby causing allosteric activation in a manner similar to the La protease. Alternatively, the moiety may stabilize the enzyme against autodegradation or other inactivation processes that may occur during the Fbg-SDS-PAGE analysis.

Because a number of the physical properties of this moiety, such as its pI and Mr, resemble those of low Mr kininogen, we explored this further. Commercial preparations of human kininogens were tested and were found to contain this activity. However, antibody studies suggest that the moiety is distinct from the high or low Mr kininogens. Thus the molecule may be a molecule that is unrelated to kininogens but that which co-purifies with them. Alternatively, it may be an immunologically distinct but related molecule. Other observations suggest that it may be a variant of serum albumin. Although we have not yet identified the molecule, we wish to ascertain whether it is taken up by trypanosomes and regulates their proteolytic activity *in vivo*. In order to measure the endocytosis of this moiety, we need to set up appropriate endocytotic assays. We have done this by using fluorescently labelled protein that can be monitored quantitatively by spectrofluorometry and qualitatively by microscopy. However, our observations have been complicated by the fact that the uptake of rhodamine-BSA, and other molecules, by trypanosomes in vitro is not always reproducible (J. Lonsdale-Eccles and P. Webster, unpublished observations). Definitive quantitative measurements of endocytosis must therefore await the resolution of this difficulty. Nevertheless, rhodamine-BSA is taken up in a time, temperature and dose dependent manner into a distinct region between the nucleus and flagellar pocket in the area previously suggested to be that in which the endocytotic network is located.\(^\text{16,17}\) Regardless of its identity, the molecule is a potential candidate to regulate trypanopain activity *in vivo* and our current studies are aimed at elucidating whether it plays such a role.

**References**


Figure 1. Dose dependent uptake of DFP by *T. brucei*. The parasites (5 × 10⁸) were incubated in 1 mL PSG with various amounts of DFP (1.5 - 25 μL). After 45 min ice-cold TCA was added to give a final concentration of 10% TCA. The precipitate was washed twice with 500 μL 10% TCA and four times with ethanol. The samples were dried and then resuspended in 250 μL SDS-gel sample buffer. 50 μL aliquots were subjected to scintillation counting.
Figure 2. SDS-PAGE analysis of DFP-labelled proteins. Samples were prepared essentially as described in Figure 1 except that the trypanosomes were pre-incubated for 45 min in the presence and absence of 1 mM chloromethylketones. The samples were subjected to SDS-PAGE, Coomasie brilliant blue staining, and fluorography. The Coomasie stained gel (not shown) demonstrated an equal loading of all samples, except for Lane 4, which had approximately half the amount of protein. Lanes 1-3, intact trypanosomes; Lanes 4-7, trypanosomes lysates; Lanes 1-3, intact trypanosomes; Lanes 4-7, trypanosome lysates.
Mechanisms for sorting ligands and receptors following endocytosis

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pH of endosomal compartments
Vesicle fusion and separation on the endocytic pathway
References

Mammalian cells have very efficient mechanisms for routing endocytosed molecules to various cellular destinations following internalization. For example, many receptors are recycled back to the cell surface while the ligands they carried into the cell are delivered to lysosomes and degraded. We have studied the mechanisms for this sorting in cultured fibroblasts, primarily Chinese hamster ovary (CHO) cells. In our studies we have emphasized the use of quantitative fluorescence microscopy and digital image processing. This has provided a powerful method for looking at endocytic processes in living cells.

Using fluorescein-labelled endocytic probes, we have measured the pH of various endocytic compartments. We have found that endosomes maintain an acidic pH and that different types of endosomes are regulated to different pH values. This acidification is required for several of the processes associated with endocytosis, including release of several ligands from receptors, dissociation of iron from transferrin, and penetration of some viruses and toxins into the cytosol.

More recently, we have developed two types of fluorescence assay for fusion of endocytic vesicles. Both methods indicate that endosomes fuse actively for several minutes after internalization. The fusion competence of the endosomes then falls off as the endosomes mature. In this paper we describe a model that can account for many features of the sorting which occurs following endocytosis. The model is based on our observations of endosome acidification and fusion.

pH of endosomal compartments

The endosomal compartments in CHO cells are illustrated schematically in Figure 1. Since the excitation of fluorescein is strongly pH dependent, the pH of endosomes can be determined by delivering fluorescein-labelled probes to the appropriate compartment and measuring the fluorescence. We have used a variety of fluorescent probes and incubation conditions to obtain the pH values shown in Figure 1.

The early endosomes were labelled by very brief incubations with fluorescein dextran, fluorescein-a 2-macroglobulin or fluorescein transferrin. At the very earliest times following endocytosis, an average endosomal pH of approximately 6.5-6.8 was measured. After a few
minutes, fluorescein-a 2-macroglobulin accumulates in large endosomes, which are easily resolved as distinct structures by fluorescence microscopy. These vesicles have an average pH of approximately 5.5. After approximately 20 minutes, a 2-macroglobulin is found in lysosomes that maintain a pH between 5.0 and 5.5 in CHO cells.

Since transferrin remains associated with its receptor as it recycles, fluorescein-transferrin can be used to measure the pH of endosomal compartments on the recycling pathway after they have diverged from the pathway leading to lysosomes. In CHO cells, we have found that the post-sorting recycling endosomes are heavily concentrated in the trans-Golgi region of the cell. The pH of these para-Golgi recycling endosomes is approximately 6.5.

From these measurements, we can establish that ligands and receptors are exposed to different acidic pH values within different types of endosomes. This pattern of acidification has several important consequences, which have been reviewed elsewhere. Many ligands such as a 2-macroglobulin, low-density lipoproteins (LDL) and epidermal growth factor will dissociate from their receptors at the mildly acidic pH, found in early endosomes. Other ligands, notably lysosomal enzymes require a more acidic pH, like that found in large endosomes, for complete dissociation. The iron that is carried into cells bound to transferrin is released at pH values below 6.0. This suggests that iron is released within the sorting endosome, since that is the lowest pH compartment on the recycling pathway. Many viruses are also brought into the cell by receptor-mediated endocytosis and pass through the same endocytic compartments. The compartment from which an enveloped virus penetrates into the cytosol may be determined by the pH at which the virus coat proteins become capable of fusing with target membranes. Only when the virus enters a sufficiently acidic endosome will it be able to cross the membrane.

**Vesicle fusion and separation on the endocytic pathway**

It is clear that ligand-receptor dissociation within the cell is obligatory in those cases where receptors are recycled as the ligand is degraded. As discussed above, the acidity of endosomes is sufficient to cause the dissociation of ligands from receptors. However, at this stage both molecules are still within the same compartment. The mechanisms for segregating the ligands from receptors following dissociation have been unclear.

To observe how the process occurs in living cells, we have developed methods for studying fusion and separation of vesicles in living cells. In one assay, endosomes are sequentially loaded with fluorescein-labelled ligands, followed by anti-fluorescein antibodies, which quench fluorescence. Endosome fusion is monitored by loss of fluorescence intensity. In a second method, we use very sensitive image intensifiers and digital image analysis to measure the fluorescence intensity of individual endosomes.

The picture that emerges from these studies may be summarized as follows (see Figure 2). For several minutes after internalization, endosomes fuse actively. For non-recycling components (e.g., LDL) this fusion results in a very large increase in the number of molecules per endosome. Histograms of endosome intensities are shown in Figure 3. We estimate that LDL per endosome increases approximately 40 fold within endosomes. In contrast, there is only a small increase in the number of recycling components per endosome. For example, we find that the transferrin per endosome increases about 2-3 fold. This indicates that transferrin is being removed as LDL continues to accumulate. We have modeled this process as a repeated series of fractionations, analogous to repeated distillation steps. If each cycle removes a higher fraction of transferrin than LDL, then repetition of the cycle can result in very efficient sorting of transferrin from LDL. Results of sample calculations are shown in Figure 4.
The values of $F$ are the fraction of molecules removed from a sorting endosome at each segregation step. For recycling molecules, $F$ values near 0.2 would lead to 90% efficient recycling within 40 cycles. The maximum accumulation would be 4 times the number of molecules in an early endosome. This is close to our observed values for transferrin. For ligands that are retained in endosomes, $F$ values near 0.01 are more appropriate. In this case, accumulation is nearly linear and only about 20% of the ligands would be recycled after 40 cycles. This is close to the observed properties for ligands such as LDL or a 2-macroglobulin. After 5-10 minutes, the endosomes no longer fuse with incoming vesicles. This would correspond to a time at which they begin to mature into lysosomes.

The geometry of the sorting endosome is well-suited for carrying out this type of fractionation. Acid-releasable ligands are distributed throughout the volume, whereas recycling components are distributed along the membrane surface. The tubular extensions of endosomes have a high surface to volume ratio. Fusion and budding of these tubular extensions would provide a simple mechanism for carrying out the repeated fractionations described in this model.

References

Figure 1. The figure illustrates pathways taken by $\alpha_2$M (a), diphtheria toxin (D), transferrin (T) and its bound iron (f), and lysosomal enzymes bearing mannose 6-phosphate (M). The receptors for $\alpha_2$M (aR), transferrin (TR), and lysosomal enzymes (MR) are also shown. After binding at the cell surface and concentration in coated pits, these ligands are internalized into small vesicular and tubular "early endosomes". The ligands and receptors move to larger, more vesicular endosomes. The large endosomes include structures termed "endocytic vesicles", "receptosomes", "late endosomes" and "multivesicular bodies". The "sorting endosome" is similar to the structure termed CURL (compartment of uncoupling receptor and ligand) that was described by Geuze and coworkers in hepatoma cells. The late, prelysosomal endosomes are the last organelle prior to lysosomes. Tf and the recycling receptors are resumed to the cell surface via "recycling endosomes located near the Golgi complex.

Figure 2. Illustrative model of repeated fractionations. Early endosomes containing LDL (L) and transferrin (T) fuse with sorting endosomes. In the model, the LDL distributes according to the volume between the lumen and the tubular extensions. This figure...
shows the distribution with 10% in the tubular extension ($F_L = 0.1$). At the same time 50% of the transferrin is distributed into the tubular extensions ($F_T = 0.5$). When the tubular extensions pinch off transferrin and LDL are trapped in the lumen of the sorting endosome or the recycling endosome. The figure shows that after three repetitions of this cycle, 75% of the transferrin is recycled whereas 83% of the LDL remains in the sorting endosome.

Figure 3. Histograms of endosome intensities. CHO cells, line TRVb1, were incubated with Rhodamine transferrin or Dil-labelled LDL for 2 minutes or 20 minutes at 37 °C. The brightness of individual endosomes was determined by digital image processing, and histograms of the intensity within each range were prepared. It can be seen that the amount of LDL per endosome increases approximately 40-fold while the transferrin content increases only slightly. This would be predicted from the type of process illustrated in Figure 2.
Figure 4. Ligand accumulations and recycling efficiency. The properties of sorting systems similar to that shown in Figure 2 were calculated for different values of F, the fraction of molecules contained in the tubular extension. Values of F near 0.2 produce a rapid saturation of the ligand content per endosome (A), and recycling efficiency near 90% is achieved after 40 cycles of fusion and segregation (C). This type of behavior is comparable to observations with transferrin. For F values near 0.02, large increases in the ligand concentration per endosome occur (B) and little of the ligand is recycled (D). This type of behaviour is seen with LDL.
Cytochemical studies on the stability of the lysosomal membrane in relation to cell function using molluscan digestive cells as a model system

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A major function of lysosomes is the autophagic turnover of cytoplasmic components, including organelles and proteins. The precise mechanisms for the uptake of proteins are not clearly identified, although there are indications that both microautophagy and macroautophagy are involved. There is considerable but mainly circumstantial evidence that reduced lysosomal membrane stability results in enhanced catabolic activity and vacuolar fusion. In view, therefore, of the importance of the lysosomal system in turnover of cytoplasmic components, and its involvement in many pathological responses, it is important to establish the relationship between lysosomal stability and lysosomal function.

Structure-linked latency of lysosomal enzymes is a complex phenomenon involving several factors, including membrane permeability and the internal associations of the hydrolases with structural components. It is well established that many agents such as various disease conditions, stress, hormones and drugs can induce destabilizing alterations in lysosomes, resulting in reduced hydrolase latency. However, the relationship between altered physiological states or pathological conditions and lower order events involving hydrolase latency and protein catabolism is seldom clear, and it is often difficult to determine whether functional changes in lysosomes are a cause or an effect of cellular dysfunction.

In our attempts to test this relationship we have used predominantly cytochemical studies on molluscan digestive cells from the hepatopancreas or digestive gland as a model system. Many molluscan cell types are particularly rich in lysosomes, which are involved in processes of digestion, excretion, immunity and autophagic mobilization of nutrient reserves. This relative abundance of lysosomes and the obvious dependence of these cells on lysosomal processes makes them suitable models for the investigation of functional relationships.

In this respect, singular attention has been paid to the digestive cells, which are in fact multifunctional. These cells are involved in the pinocytic uptake of food and its subsequent digestion in secondary lysosomes. However, digestive cells also have a degree of functional analogy with vertebrate liver cells (hepatocytes) in that they store glycogen and lipid, and are the site of major physiological processes in the animal, including detoxication and excretion. As well as being of considerable metabolic significance, the digestive gland is a major interface between the organism and its environment. Previous studies have clearly demonstrated that the cells of this organ are highly responsive to toxic chemical pollutants. Most of these responses apparently involve both structural and functional alterations in
lysosomes, although it is probably artificial to discriminate between them. We have been particularly interested in the identification of causal relationships in the responses of marine molluscs to environmental stress and establishing a conceptual framework for the chain of events from environmental stimulus to consequences for the organism. Our approach has been to examine responses to stress and various drugs and xenobiotics at several levels of biological organization, namely from the biochemical and subcellular to that of the whole animal.

This report summarizes progress in this area, with particular emphasis on the cellular consequences of experimentally induced alterations in lysosomes. In attempting to establish functional links we have used experimental manipulation of digestive cells in order to characterize their lysosomal and cellular reactions. In this context, H.K. Hawkins has subdivided lysosomal reactions into three basic categories, namely, changes in membrane permeability, in vacuolar fusion events and in lysosomal contents.3

Lysosomal reactions in molluscs fall into these three classes, which often may have considerable overlap. We will now consider these reactions in more detail.

Changes in lysosomal stability are induced by a variety of in vivo experimental treatments of mussels. These include elevated temperature, hypoxia, salinity increase, certain sex steroids and lipophilic xenobiotics.6 All of these treatments result in a decrease in cytochemically determined stability of lysosomes, based on hydrolase latency, and this has been confirmed biochemically in selected experiments. These effects are believed to involve an increase in the permeability of the lysosomal membrane because they are frequently reversible by cortisol treatment, which is a recognized membrane stabilizer (Figure 1).8,9

Reductions in lysosomal stability in digestive cells have frequently been demonstrated to be reversible during the course of adaptation to particular stimuli such as salinity increase; this suggests that such changes may have an adaptive physiological role.10,11 The possible consequences of destabilizing alterations in lysosomal membranes have been investigated in a number of experimental conditions, for instance, treatment of mussels with 17 β-estradiol results in a rapid destabilization of digestive cell lysosomes (Figure 1).9 This is accompanied by an increase in the formation of tertiary lysosomes containing lipofuscin, which may be indicative of augmented autophagy.9

Salinity increase also results in a rapid decrease in lysosomal stability and evidence of enhanced autophagy.10,11,12 Mussels are known to osmoregulate intracellularly by increasing the concentration of cytosolic free amino acids.10,11 These are probably generated by enhanced protein degradation and this hypothesis is supported by evidence of increased amino acid concentrations in fractionated lysosomes.11 The implication is that decreased lysosomal stability is involved in the activation of lysosomes for the autophagic degradation of cytoplasmic proteins.

More recent studies have used the polycyclic aromatic hydrocarbon (PAH) phenanthrene as a model destabilizer of digestive cell lysosomes.13,14 Digestive cells have a relatively low cytochrome P-450 content, resulting in a low rate of metabolism of PAHs and other lipophilic drugs.7 The consequence is that PAHs accumulate in these cells and there is evidence that the lysosomes are a major site for their sequestration.15 The response relationship between tissue concentration of phenanthrene and lysosomal stability has been determined and this has a sigmoidal form indicating an "all or nothing" type of response.13,14 This finding suggests that at a particular threshold level of incorporation of phenanthrene into the lysosomes, there is a "catastrophic" alteration in membrane organization involving permeability/fluidity changes resulting in the reduced stability of latent β-N-acetylhexosaminidase and β-glucuronidase.
Concomitant determinations were made of the loss of $^{14}$C-label from pre-labeled cytosolic proteins in the digestive gland cells over a concentration range of phenanthrene. The results indicated that there was an increased loss of $^{14}$C-label only from cells in which the lysosomes were destabilized (Figure 2). As the possibility of reincorporation of $^{14}$C-label due to protein synthesis had been minimized, this finding was consistent with enhanced catabolism of cytosolic proteins. In fact, both lysosomal membrane stability and concentration of $^{14}$C-label cytosolic protein were significantly correlated ($r = 5, p = 0.001$, two-tailed test).

Other changes in lysosomes frequently accompany the measured decrease in stability. These include increase fusion of vacuolar components, lysosomal swelling, increased neutral lipid and lipofuscin content and increased numbers of tertiary lysosomes (Figure 3). These changes are all consistent with the hypothesis of increased autophagy and catabolic activity resulting from increased lysosomal fragility. Ultrastructural evidence of increased architectural fragility of lysosomal membranes and enhanced fusion with other vesicular components lends further support to this hypothesis. Furthermore, evidence of enhanced microautophagy and leakage of hydrolase into the cytosol following treatment with PAHs has been tentatively linked with reduction of membrane stability.

Additional support for the hypothesis that enhanced catabolic activity is linked with lysosomal fragility is provided by evidence of atrophic alterations in the digestive cells following lysosomal destabilization.

In conclusion, we can say that although the evidence of increased protein catabolism associated with lysosomal fragility is still correlational, there is considerable supportive evidence that increased membrane fragility does in fact result in enhanced autophagy leading to atrophy of the digestive cell.

**Acknowledgement**

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**References**


Figure 1. The effects of injections of sex steroids (100 µl 5 × 10⁻⁷ M) and cortisol (100 µl 10⁻² M) on the labilisation period of lysosomal hexosaminidase after 2 h in Mytilus edulis (mean ± SE).
Figure 2A. The effect of phenanthrene concentration in seawater on specific activity of $^{14}$C-labelled cytosolic proteins (as % of control values) in the midgut gland (each point is the mean ± SE of at least five replicate experiments).
Figure 2B. The effect of phenanthrene concentration in seawater on lysosomal stability in digestive cells (each point is the mean ± SE of five replicate experiments, each experimental treatment contained five animals). Exact probability values are given in the figures where values differ significantly from those of the control. These are shown vertically above the appropriate data points.\textsuperscript{13}

Figure 3. Results of stereological analyses of lysosomes in the digestive cells of mussels. Controls (C) and experimental (E) mussels exposed to water accommodated fraction (PAH-rich, 30 ppb total hydrocarbon) of North Sea crude oil were sampled after 34 and 103 days. Bar height gives a point estimate of the relevant parameter; the
The associated interval estimates are 95% CI/Ö 2.
Formation of disulphide linked oligomers of the HN glycoprotein of Newcastle disease virus

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Intermolecular disulphide bonds form intracellularly
Oligomer formation and intracellular transport
Expression of the HN protein using a retroviral vector
Disulphide-linked oligomer formation early in infection
Conclusions
References

Plasma membrane glycoproteins are transported to the cell surface by cellular pathways that begin with the insertion of the nascent protein into the membrane of the rough endoplasmic reticulum (RER) and proceed with the transport of the protein through the Golgi membranes to the plasma membrane.1 During transport, glycoproteins are subjected to a variety of co- and post-translational modifications. In addition, the nascent protein must fold properly for full activity and possibly transport to the cell surface.2,3,4 One component of proper folding is the formation of intra- and, sometimes, intermolecular disulphide bonds.5,6,7 Current evidence suggests that disulphide bond formation is mediated by protein disulphide isomerase (PDI) located in the lumen of the rough endoplasmic reticulum.5,7

The glycoproteins of simple enveloped viruses have been useful in the study of the transport, modification and folding of plasma membrane glycoproteins. The HN protein of Newcastle disease virus (NDV), a paramyxovirus, has been used here to explore the formation of intermolecular disulphide bonds and the role this sort of post-translational modification plays in the intracellular processing of a glycoprotein. The HN glycoprotein is a type 2 glycoprotein,8 that is, the protein has no cleavable signal sequence but rather a bifunctional hydrophobic region near the amino terminus which serves as both a signal sequence and a membrane anchor. The amino terminus of the protein is the cytoplasmic tail while the carboxy terminus is transported across the membrane. It has been well documented that the HN glycoproteins of some paramyxoviruses, including the AV strain of NDV, form disulphide linked homooligomers.9 Indeed, as shown in Figure 1, virion associated HN protein is exclusively in the form of disulphide linked oligomers. The monomeric form of HN protein seen in the presence of reducing agent is absent when proteins are electrophoresed without reducing agent. Without reduction of virion proteins, HN is resolved as a high molecular weight band near the top of the gel (HN0). Evidence is presented here that the NDV HN disulphide linked oligomers form in the late stages of residence in the RER. However, not all nascent HN protein forms of oligomers and oligomer formation is not required for transport of the protein to the cell surface. Indeed, evidence is presented that the level of oligomer formation depends upon the concentration of the protein in cells.
Intermolecular disulphide bonds form intracellularly

To explore the formation of intermolecular disulphide bonds, infected cells were pulse labelled (5 min) or pulse labelled and then chased with non-radioactive methionine in the presence of cycloheximide. Proteins present in the cell extracts were resolved on polyacrylamide gels in the presence or absence of reducing agent (Figure 2). We have previously found that the HN protein is transported through the cell extremely slowly.\(^{10}\) Pulse labelled HN reaches the cell surface with a t\(_{1/2}\) of 78 min. We also found that 100% of cell associated pulse-labelled HN protein reaches the cell surface after approximately 2.5 hours of chase.\(^{10}\) Therefore, chase times were extended to four hours. It is clear that the oligomer does not begin to appear until 15 min of chase and continues to increase in amount up to 2 to 3 hours of chase.

To determine the intracellular location of the formation of the oligomer, use was made of inhibitors that block glycoprotein migration at various points in the cell. CCCP, an inhibitor of oxidative phosphorylation, blocks any energy-requiring process such as migration of protein from the RER.\(^{11}\) Figure 3A, lanes 5 and 6, shows that if CCCP is present during the non-radioactive chase, the disulphide linked oligomer does not form. Monensin, a sodium ionophore, is reported to interfere with the transport of most membrane glycoproteins by preventing their exit from the medial Golgi membranes.\(^{12}\) Clearly, the HN oligomer forms in the presence of monensin (Figure 3B, Lanes 6, 7 and 8).

These results argue that the formation of the disulphide-linked oligomer of the HN protein occurs posttranslationally prior to the exit from the medial Golgi membranes. The results with CCCP suggest that the formation of the disulphide bonds either requires energy in the RER or that the oligomer forms after the protein leaves the RER. It has been shown that when cells are incubated at 15°C, glycoprotein transport from the RER is blocked.\(^{13}\) When pulse-labelled NDV-infected cells are subjected to a nonradioactive chase at 15°C, the disulphide-linked oligomer forms (not shown). Thus, it is likely that the formation of the HN oligomer is an energy-requiring process that occurs in the RER, a finding consistent with the proposed location of PDI in eucaryotic cells.\(^{15}\)

Oligomer formation and intracellular transport

A key to the understanding of the intermolecular disulphide bond formation in intracellular transport of the HN protein is the efficiency of this post-translational modification. If this modification is required for intracellular transport of the protein, all HN protein that reaches the cell surface should be in the form of disulphide-linked oligomers. It is clear from Figure 2 that not all pulse labelled HN protein is found in oligomers after prolonged chase periods. This finding was confirmed by immunoprecipitating the HN protein from these extracts with antibody specific for the HN protein (see Figure 7). Thus, we asked if the monomeric form of the protein can be found at the cell surface. We have previously devised an assay to isolate only cell surface glycoproteins using polyclonal antisera directed against NDV proteins.\(^{10}\) We have also adapted the assay for monoclonal antibody directed against the HN protein (Morrison and McGinnes, in preparation). Using this assay, and isolating the cell surface HN protein under non-reducing conditions, it is clear that a significant amount of cell surface HN is in the form of monomers (Figure 4).

It is possible that monomeric forms of the HN protein are less efficiently incorporated into the plasma membranes. To determine the kinetics of insertion into the cell surface of the two forms of HN protein, cell surface molecules were isolated at various times after the onset of the chase and resolved on gels. The amount of the two forms of the HN protein were quantitated with a densitometer. Figure 5 shows that the kinetics of appearance at the cell surface, as well as the release, of the monomer and the oligomer are very similar. Thus, the
The monomeric form of the HN protein is transported to the cell surface at a rate similar to that of the disulphide-linked form.

**Expression of the HN protein using a retroviral vector**

A cloned copy of the HN gene was expressed in chick cells to explore further the processing of this protein. The expression vector used for these studies is a retrovirus derived from Rous sarcoma virus. This virus is replication competent but with the sarc gene deleted. In the place of the sarc gene is a C1a I site for insertion of foreign DNA. Insertion in one direction allows for the expression of the sense version of the gene, while the opposite direction allows for the transcription of antisense sequences. The cDNA of the HN gene was inserted into a plasmid containing a DNA copy of the retrovirus and chick embryo fibroblasts were transfected with the DNA. Since the virus is replication competent, transfected cells will release virus, allowing for the spread of the infection throughout the entire culture. Thus all cells in the culture receive the HN gene. Figure 6 shows that the cells transfected with the retrovirus containing the sense version of the HN gene (RHN) express the HN protein, whereas cells that received DNA with the HN gene inserted in the antisense direction (R antiHN) do not contain the protein. The level of HN expression is approximately 5-10% that seen in NDV-infected cells 6 to 8 hours after infection.

The HN protein expressed from the retrovirus vector is transported to the cell surface. To assay for cell surface expression, two different assays were used. First, antibodies were used to isolate specifically cell surface molecules as described above. RHN cells contain HN at the cell surface while RSV or R antiHN cells are negative (not shown). A second assay for cell surface expression of the HN protein takes advantage of the ability of the HN protein to attach to red blood cells. Cells expressing HN on their surfaces will adsorb red blood cells. Table 1 shows a quantitation of haemadsorption using ND-infected cells. At various times after infection, red blood cells are added to intact cells, incubated once, and unbound blood cells washed away. Bound red blood cells are lysed and the amount of released haemoglobin determined in order to quantitate the amount of red blood cell binding. There is negligible binding early in infection, while binding increases with time, reflecting the presence of the HN protein on infected cell surfaces. Similar assays were done with the RHN cells. Significant binding of red blood cells \((6 \times 10^6)\) was observed while the antisense expressing cells were negative.

A surprising result was that the ratio of the monomeric form and disulphide-linked oligomers present in these HN expressing cells was quite different than that observed in infected cells (Figure 7, compare top and middle panels). In this experiment, NDV infected cells contained 70% of the HN protein in the form of disulphide-linked oligomer, whereas the retrovirus-infected cells contained 10% in the form of a disulphide-linked oligomer. In addition, the vast majority of cell surface HN protein is in the form of a monomer (not shown). There are two possible explanations for the reduced level of disulphide-linked oligomers in these cells. First, it is possible that the presence of another viral protein in the infected cells promotes the formation of disulphide-linked oligomers. A second possibility is suggested by the fact that the level of HN protein expression from the retrovirus vector is lower than that in an actively infected cell. Perhaps disulphide-linked oligomers form efficiently only after the HN proteins reach a critical concentration in the cell.

**Disulphide-linked oligomer formation early in infection**

If it is the concentration of protein in a cell that promotes the formation of disulphide-linked oligomers, then monomer formation should be favoured very early in an infected cell when the concentration of viral proteins is relatively low. To test this possibility, infected cells were subjected to a pulse chase at 3 to 5 hours post-infection, in contrast to the usual time of 5 to 7
hours post-infection. The ratio of monomers and disulphide-linked oligomers was determined. Figure 7, bottom panel, shows a densitometer scan of the resulting auto-radiograph. At the early time point, most of the HN protein is in the form of a monomer (75%). However, later in infection, oligomer formation is favoured (Figure 7, top panel). This result argues that it is the concentration of the protein that determines the ratio of the monomer and the disulphide-linked oligomer.

Conclusions

One recently described post-translational modification of two viral glycoproteins, the influenza haemagglutinin protein HA and the vesicular stomatitis virus glycoprotein G, is oligomerization of the protein.2,3,4 The influenza haemagglutinin protein forms trimers and the vesicular stomatitis virus glycoprotein forms dimers and trimers soon after synthesis in the rough endoplasmic reticulum. Evidence has been presented that the formation of these homo-oligomers is essential for transport of the glycoproteins out of the RER. Thus, an obvious question is the universality of this requirement. It is clear from the results presented here that formation of the disulphide-linked oligomer of the NDV HN protein is not a prerequisite for transport from the RER. Significant amounts of the monomeric form of the protein can be detected at the cell surface. In addition, the amount of disulphide-linked oligomer in cells seems to depend upon the level of expression of the protein. Thus, the formation of oligomers may not be a universal requirement for the intracellular processing of viral glycoproteins.

While not all HN protein forms disulphide-linked oligomers, virion associated protein is exclusively in this form. Thus the fate of cell surface monomeric HN protein is of interest. It is possible that monomeric forms incorporated into virions rapidly form oligomers during budding. Alternatively, it is possible that monomers are not used in virion formation and may be endocytosed and degraded. This latter possibility raises interesting considerations for viral infections under conditions when gene expression is limited, such as persistent infections.

References


Table 1. Haemadsorption of NDV-infected cells

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<th>Time after infection (hrs)</th>
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Figure 1. Virion proteins electrophoresed in the presence (+) or absence (-) of β mercaptoethanol.

Figure 2. Pulse-chase analysis of infected cells. Cells infected for 5 hours were pulse-labelled with ³⁵S methionine (P) for 5 min or pulse-labelled and then chased for various
times. Total cytoplasmic extracts were electrophoresed on 10% polyacrylamide gels. The lanes from left to right are 0, 5, 10, 15, 60, 90, 120, 180 and 240 minutes respectively.

Figure 3. CCP and monensin treated infected cells. Panel A: cells were pulse-labelled for 5 min and then chased for 1 hr (Lanes 2 and 5) or 2 hrs (Lanes 3 and 6) in the absence (Lanes 2 and 3) or presence of CCCP (Lanes 5 and 6) Panel B: Pulse-labelled cell extracts are shown in Lanes 1-4. Pulse-chase extracts are shown in Lanes 5-8. Increasing concentrations of monensin ($10^{-7}$, $10^{-6}$, $10^{-5}$ M) were used (Lanes 2-4, 6-8).

Figure 4. Cell surface HN protein electrophoresed in the presence or absence of b mercaptoethanol. Cell surface molecules were isolated from cells chased for 2.5 hours as previously described. M, marker proteins.
Figure 5. The kinetics of appearance and release of cell surface HN monomers and oligomers. Cells pulse-labelled for 5 min were chased for various lengths of time and cell surface HN isolated and quantitated by densitometer scans of autoradiographs of polyacrylamide gels.

Figure 6. Proteins immunoprecipitated from retrovirus-infected cell extracts with anti-HN monoclonal antibody. R, retrovirus alone; RHN, retrovirus containing HN gene; R anti-HN, retrovirus containing antisense HN gene. Electrophoresis was in the presence
of b mercaptoethanol.

Figure 7. HN protein in NDV-infected cell extracts and retrovirus-infected cell extracts. Cells were pulse-labelled for 5 min and chased for 2 hrs. HN protein present in the resulting cytoplasmic extracts was immunoprecipitated with monoclonal antibody against HN protein. The figure shows densitometer scans of autoradiographs of polyacrylamide gels of the immunoprecipitate. M, monomer; O, oligomer. Top panel: NDV-infected cells chased from 5 to 7 hours post infection. Middle panel: retrovirus containing the HN gene, 2 hour chase. Bottom panel: NDV-infected cells chased from 3 to 5 hours post-infection.
Theileria parva, a tick-transmitted protozoan parasite, is the causative agent of theileriosis, an often fatal lymphoproliferative disease of livestock in East and Central Africa. The related parasites T. annulata and T. sergenti, cause similar diseases in the Middle East/India and the Far East, respectively. Although T. parva sporozoites may enter a variety of bovine cell types, they appear to be able to survive only in subpopulations of lymphocytes in which the parasite differentiates into a multinucleate schizont. Theileria schizonts are unique among protozoan parasites in that they induce lymphoblastogenesis and clonal expansion of quiescent target cells. Regardless of pre-infection phenotype, the Theileria-infected cells acquire some surface antigens characteristic of proliferating bovine T cells. Since such cells are immortalized and can easily be propagated in vitro, exhibit cellular pleomorphism and appear to acquire a variety of other alterations in surface phenotype, Theileria-infected cells have been considered transformed. However, such terminology has been considered misleading since it implied an oncogenic aetiology and since the infection is curable with administration of appropriate antibiotics.

Protein kinase activation in Theileria-infected cells

Ample evidence has been presented to implicate phosphorylation/dephosphorylation in the modulation of a large number of processes vital to the life of a cell. Among these are cellular differentiation, signal transduction and cellular proliferation. Cells can proliferate in response to external signals as well as to intracellular events. Membrane receptors for several polypeptide hormones and growth factors have been shown to have endogenous tyrosine-specific protein kinase activity. Upon receptor engagement by ligand, activation of the receptor kinase occurs, which initiates a series of modifications of specific target molecules, which are thought to constitute intracellular pathways for cellular growth (for review, see 8). Under normal circumstances, when a signal for cell growth ceases, homeostatic regulation ensures inactivation of important molecules or the reversion of their levels to those obtained at quiescence. It is thus envisaged that uncontrolled cellular growth could occur if important molecules regulating cellular proliferation are constitutively produced or if because of structural alterations, such molecules cease to be responsive to physiological control.

The important roles played by protein kinases in growth of cells stimulated us to determine if any differences existed between the activities of these enzymes in cloned bovine lymphocytes before and after infection with Theileria sporozoites. Supernatant and particulate fractions (100,000 × g) prepared from IL-2 maintained, con A stimulated or normal peripheral blood
lymophocytes (PBL) were used as control for those from *Theileria*-infected cells. Most enzymatic activity was in the 100,000 × g particular fractions and was capable of phosphorylating both endogenous and exogenous substrates.

Analysis of target amino acids residues\(^1\) and kinase co-factor requirements showed that the *Theileria*-associated kinase(s) were cyclic nucleotide independent, Ca\(^{++}\) calmodulin insensitive and phosphorylated serine/threonine, rather than tyrosine, residues on endogenous protein substrates. Among a large number of exogenous substrates examined, phosvitin, glycogen synthase and a wide variety of casein variants were the best substrates.

The susceptibility of the *Theileria*-associated kinases to inhibition by glycosaminoglycans, 2,3-bisphosphoglycerate, pyridoxal 5'-phosphate and to stimulation by polyamines suggests that the dominant protein kinase activity in *Theileria*-infected cells is casein kinase II-like.\(^6,7\)

Furthermore, employing anti-bovine casein kinase II antibody,\(^5\) we have shown that the *Theileria*-infected cells have markedly increased amounts of casein kinase II antigen. Also the enzymatic activity is susceptible to partial inhibition by this antibody. Interestingly enough, in addition to a \(a\) and \(b\) subunits of the bovine casein kinase II,\(^5,17,18\) we also see strongly reacting antigens in the Mr range of 15-20 kDa. The latter have only been seen so far in *Theileria*-infected cells. The latter findings are in agreement with our finding a casein kinase II-like enzymatic activity in purified *Theileria*-macroschizont preparations.\(^19\) Current efforts are directed at structural and functional characterization of the parasite enzyme.

**Conclusions**

(1) Our findings of substantial increases of casein kinase II-like enzymatic activity, as well as antigen, in *Theileria*-infected cells suggest a possible role for this enzyme in *Theileria*-induced cellular proliferation. This suggestion is supported by recent findings showing:

(a) Brisk activation of casein kinase II in response to hormonal stimulation of cells \textit{in vitro}.\(^17\)

(b) Elevation of casein kinase II activity and antigen in differentiating cells.\(^18\)

(c) Significant structural homologies between casein kinase II from various sources and the products of the cell cycle controlling genes in yeast and other species.\(^12,15,16\)

(2) Induction of uncontrolled cellular proliferation by an intracellular parasite may thus obviate the need to invoke exogenous factors. The *Theileria* schizont may activate a pathway that the cell engages in response to normal growth signals. Uncontrolled growth may thus occur if *Theileria* drives the lymphocytes to constitutively produce normal growth signal-transducing molecules or, alternatively, if *Theileria*-derived analogs of molecular constituents of intracellular pathways for growth may not be susceptible to modulation by host-cell elements.

**References**


Assembly and intracellular transport of a multichain protein: human fibrinogen

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Early studies
Intracellular forms of fibrinogen
Order of chain assembly
Enzymatic mechanism of chain assembly
Secretion of nascent fibrinogen
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Fibrinogen is a large molecular weight (Mr 340,000) glycoprotein present in plasma. Its primary function is in blood clotting; it is cleaved by thrombin, and is transformed to fibrin clots and specifically binds to receptors on platelets and regulates their aggregation. Human plasma fibrinogen is a dimer with each half-molecule composed of three different polypeptides; Aa (Mr 66,000, 610 amino acids), Bb (Mr 54,000, 461 amino acids) and t (Mr 48,000, 411 amino acids). The half-molecules of the dimer are held together by symmetrical disulphide bonds between two Aa chains (at Aa -28) and two t chains (at t -8 and t -9). In addition, fibrinogen contains a number of inter- and intrachain disulphide linkages. The 6 fibrinogen polypeptides are held together by 29 disulphide bonds and the protein does not have free sulphydyl groups.

The fibrinogen chains contain several prosthetic groups. Both the Bb and t chain contain asparagine-linked carbohydrates, ester-bound phosphate occurs in the Aa chain and tyrosine-O-sulfates in the Bb chain. There are two non-allelic forms of the t chain. In the t chain the 4 carboxy-terminal amino acids of the t chain are replaced by 20 amino acids.1,2,3

The complete molecule appears by electron microscopy to form a trinodal structure linked by slender rope-like strands. Studies in which both image processing of electron micrographs and analysis of low resolution X-ray crystallography were used extends this picture. It is thought that the central domain of fibrinogen contains the two amino-terminal portions of the half-molecule and the two end domains are composed of the carboxy-termini of the Bb and t chains. The carboxy-termini of the two larger Aa chains are believed to fold back to form an additional central domain.4

The fibrinogen genes have been localized in the long arm of chromosome 4 and the three fibrinogen genes are linked in the order of t,a,b with the Bb gene in the opposite orientation. Complete DNA sequences of the human t and partial sequences of the human Bb gene and rat Bb t genes are known. There is little similarity in the three genes. On stimulation of fibrinogen synthesis, during an acute phase response, there is a coordinate increase in levels of all three mRNAs and this is accompanied by changes in the transcription activity of each of
the three fibrinogen genes. This indicates that similar mechanisms regulate the activity of the three fibrinogen genes.5

Fibrinogen is primarily synthesized by hepatocytes, but megakaryocytes are also capable of producing small amounts of fibrinogen. The production of fibrinogen by hepatocytes is probably controlled by humoral agents. One mechanism, which is attractive and has received much experimental attention, is that some degradation products of fibrinogen and fibrin elicit the formation of a small peptide (about 30,000 daltons) from monocytes and/or Kupffer cells, which in turn stimulates hepatocytes to produce more fibrinogen.6,7 The stimulatory agent, first termed hepatocyte stimulating factor, is probably identical to interferon β 2- B cell differentiation factor 2.8

Thus, fibrinogen is a multi-chain protein held together by an intricate array of disulphide bonds and the three chains are intertwined in a specific pattern giving fibrinogen its unique properties, which allow it to circulate in the blood awaiting signals to perform its haemostatic functions. How this complex multi-chain protein is assembled intracellularly and what regulates its production and secretion are the questions which our studies aim to answer.

**Early studies**

The separate chains of bovine,9 dog10 and rat11 fibrinogen are synthesized by mRNA-dependent membrane-free translation systems as larger ("signal") precursors. This, together with molecular cloning of cDNA for the Aa, Bb and t chains of fibrinogen, showed that the three chains are synthesized from separate mRNAs. In vivo studies in dogs indicated that the dimeric molecule is quickly assembled in the rough endoplasmic reticulum (RER),12 but a time course study of secretion of rabbit fibrinogen suggested that the three chains are not immediately assembled prior to secretion. Newly secreted rabbit fibrinogen first contained nascent radioactive Bb chains, followed later by Aa and t chains.13 Pulse-chase experiments with Hep G2 cells confirmed that newly secreted fibrinogen contains higher specific radioactivity in the Bb chain than in Aa and t chains, again suggesting that pools of Aa and t chains exist intracellularly.14

Most of our knowledge of fibrinogen assembly relies on the results of experiments performed with a human hepatocellular carcinoma, the Hep-G2 cell.15 Hep-G2 cells were pulse-labelled with L-[35S] methionine and "chase" incubated with non-radioactive L-methionine. At various times the cells and the incubation medium were separated and fibrinogen and fibrinogen-related materials were isolated by immunoprecipitation with a polyclonal antibody that recognizes intact fibrinogen as well as its component chains. Prior to immunoprecipitation the cells were homogenized in the presence of 0.2 M iodo-acetamide to block free sulphydryl groups and minimize further disulphide interactions. The fibrinogen compounds were separated by SDS-PAGE and detected by autoradiography. In some experiments the fibrinogen-related compounds were excised from the gel and reduced and re-electrophoresed to determine the chain compositions and the specific radioactivity of the component chains.

**Intracellular forms of fibrinogen**

In non-reducing gels, eight fibrinogen-related radioactive bands were obtained. The estimated molecular weight of these proteins, as deduced from their electrophoretic mobilities on SDS-PAGE and the chain composition of each is given in Table 1.

These studies confirm the presence of excess Aa and t chains and demonstrates that most of these "extra" Aa and t chains exist as Aa - t complexes. Free t chains are present, but very few
free Aa or Bb chains.

The mechanisms by which excess Aa and t chains accumulate within Hep-G2 cells are poorly understood. Unequal synthesis of the three chains may contribute to these pools. We have determined that the initial rates of synthesis of Bb chain is less than that of Aa and t chains. This imbalance in the ratios of intracellular chains may also be due to small differences in the degradation rates of the three chains.

**Order of chain assembly**

The sequence in which the three component chains of fibrinogen interact with each other to form fibrinogen was determined by following (1) the order in which the radioactivity of pulse-labelled forms of fibrinogen decrease during the "chase" incubation (Figure 1) and (2) the radioactivities of each of the chains in the various fibrinogen-related compounds at the end of the "pulse" period and at different "chase" times. An interpretation of the results is presented in Figure 2, and for convenience we have divided the intracellular assembly of fibrinogen into 5 steps.

**Step 1.** Unequal synthesis of the three chains, and perhaps unequal rates of degradation, leads to an accumulation of excess Aa and t chains. Aa and t chains combine to form an Aa-t complex (band C) which accounts for the major intracellular form of fibrinogen-related material. Hep-G2 cells also contain free t chains. These act as intracellular "pool" forms of Aa and t chains.

**Step 2.** Fibrinogen assembly begins by the attachment of either Aa or t chains, drawn from the pool, with nascent Bb chains. This occurs while the Bb chain is not fully completed and is still attached to polysomes of the RER. For incomplete Bb chains to react with Aa and t chains the incomplete Bb chains, has to be of sufficient length to span the large ribosomal subunit and the width of the ER membrane and to expose N-terminal cysteine residues to the luminal surface of the ER. The earliest precursor forms of fibrinogen noted was "pulse" labelled incomplete Bb of between 27,000 and 32,000 daltons, complexed to either non-radioactive Aa or non-radioactive t chains. The size of the Bb polypeptide is sufficient to meet the above criteria.

**Step 3.** On completion of Bb chain synthesis, the Bb-Aa and Bb-t complexes are released into the luminal side of the ER and the remainder of the fibrinogen chain assembly occurs in the lumen of the RER.

**Step 4.** t chain is added to the nascent Bb-Aa complex and Aa is added to the nascent Bb-t, forming half-molecules of fibrinogen. The Aa and t chains are obtained from the intracellular pools. Analysis of these precursor forms from early "pulse-chase" periods show that they are composed of radioactive Bb and non-radioactive Aa and t chains.

**Step 5.** The final step in fibrinogen chain assembly is the joining of two half-molecules by the formation of symmetrical disulphide bonds between two Aa and two t chains.

All of the above steps occur in the RER. Fibrinogen at this stage is not fully glycosylated and has not yet attained its phosphate and sulphate moieties. These final touches are thought to occur in the Golgi complex, prior to secretion.
Other processing steps are probably involved in the final production of fibrinogen, cDNA cloning studies suggest that a precursor of Aα chain, with an extension at the carboxyl terminus, is a likely primary translation product, but our procedures do not allow us to distinguish between the secreted forms of Aα and the putative precursor.

That there is a pool of Aα and τ chains and that Bβ chains are directly inserted into fibrinogen precursors suggest that synthesis of the Bβ chain may be the limiting factor in fibrinogen assembly.

Fibrinogen production may be easily regulated by modulating the rate of synthesis of the Bβ chain. However, stimulation of fibrinogen synthesis in rats by defibrination results in an increase of all three fibrinogen mRNAs indicating control at the transcriptional level. Stimulation of synthesis in rabbits does not change the relative specific radioactivities of the component chains of secreted fibrinogen, indicating that increased fibrinogen synthesis does not alter the sequence of fibrinogen chain assembly.

**Enzymatic mechanism of chain assembly**

An enzyme, a protein disulphide isomerase, is present on the inner surface of the ER membrane and is particularly abundant in secretory tissues. This enzyme may catalyze both intra- and interchain disulphide interactions and can rearrange disulphide bonds in an oxidized protein and also the formation of native disulphide bonds in reduced proteins. Therefore fibrinogen precursors may exist in either reduced or oxidized forms prior to further interactions with other fibrinogen chains. Incubation of Hep-G2 cells with 14C-iodoacetamide detected only substantial free sulphydryl groups in the Aα-τ complex, with small amounts in the free τ chain.

Ordered disulphide-bond formation of fibrinogen chains is not spontaneous and requires more than added enzyme. Attempts at *in vitro* assembly, in the presence or absence of disulphide isomerase, has led only to a haphazard assembly of chains. The chains probably also need to be held in proper juxtaposition. In this regard, it should be noted that a protein in the RER, termed IgG heavy chain binding protein (BiP), binds to nascent proteins that are incompletely assembled or with malformed proteins. Immunoprecipitation of nascent fibrinogen also co-precipitates a similar 78,000-dalton protein.

**Secretion of nascent fibrinogen**

Only fully assembled fibrinogen is secreted into the medium by Hep-2 cells. None of the precursor forms of fibrinogen, the Aα-τ complex or the free τ chains, are secreted. Since free Bβ chains do not occur intracellularly, but are complexed to other chains even before they are completed, we determined the fate of free Bβ chains by transfecting a surrogate secretory cell (COS-1) with a shuttle vector pBc12BI containing full-length Bβ cDNA. The Bβ chain was expressed and not secreted. This suggests that the "signal" for secretion probably does not occur on any individual chain but resides on the intact dimeric molecule.

**References**


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Table 1. Size and chain composition of intracellular fibrinogen precursors

<table>
<thead>
<tr>
<th>Bands on SDS-PAGE</th>
<th>Estimated Mr</th>
<th>Chain composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>340,000</td>
<td>Aa, Bb, t</td>
</tr>
<tr>
<td>B</td>
<td>214,000</td>
<td>Aa, Bb, t</td>
</tr>
</tbody>
</table>
Table 2. Amount of endogenous fibrinogen-related protein detected by different procedures

<table>
<thead>
<tr>
<th></th>
<th>% of total fibrinogen antigens</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pulse-label</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>4</td>
</tr>
<tr>
<td>Band B (half-molecule)</td>
<td>10</td>
</tr>
<tr>
<td>Band C (Aa -t)</td>
<td>18</td>
</tr>
<tr>
<td>Band D(Bb -a and t)</td>
<td>15</td>
</tr>
<tr>
<td>Band E and F (incomplete + Aa or t)</td>
<td>12</td>
</tr>
<tr>
<td>Aa (free chain)</td>
<td>2</td>
</tr>
<tr>
<td>Bb (free chain)</td>
<td>3</td>
</tr>
<tr>
<td>t (free chain)</td>
<td>36</td>
</tr>
</tbody>
</table>

Hep-G2 cells were incubated with L-$^{35}$S-methionine for either 3 mins (pulse-label) or 15 h (steady state). The percentage of radioactivity in the various compounds was determined by scanning the autoradiograms with a laser densitometer and calculating the intensity profiles.

Figure 1. Pulse-chase incubation of Hep G2 cells with L-$^{35}$S methionine. On the left is an autoradiogram of fibrinogen-related materials at the end of a 3 min pulse and on the right, that at the end of a 20 min chase period.
Figure 2. Scheme for the assembly of fibrinogen.
Synthetic inactivators of serine and cysteiny1 proteases for biological application

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Use of protease specificity for inhibitor structure
Peptidyl fluoromethyl ketones
Peptidyl diazomethyl ketones
Peptidylmethyl sulphonium salts
Summary
References

The development of synthetic protease inhibitors that function by affinity-labelling offers the possibility of obtaining reagents that may be used with living systems to inactivate a target protease by covalent combination. The perturbation in the system should give some indication of the normal function of the protease. If the protease has a role in pathology, an effective inhibitor may have therapeutic value. The type of reagent we study is generally a low molecular weight peptide derivative. Irreversible combination with the target protease permits the inhibitor to resist displacement by normal substrates which are generally high affinity or present in relatively high concentration. In addition, the low molecular weight inhibitor may be made highly radioactive, permitting an analysis of the system with identification of the molecular target(s) and a correlation of the protein modification with some change in function. As the selectivity of the reagents improves, this type of application should become more common. It is already feasible with a number of proteases.

Use of protease specificity for inhibitor structure

Our work has dealt with two of the proteinase classes, the serine and cysteiny1 proteinases. (Metallo and aspartyl proteinases are insensitive to the types of reagents we study.) A knowledge of target enzyme specificity is essential for devising proteinase inhibitors that act by affinity labelling. Proteinases have an extended active centre that may combine with a substrate sequence of six or more amino acids. A familiar example is provided by the trypsin family of serine proteinases, which act on lysine and arginine residues. Individual members of this family are responsive to amino acid residues preceding the lysine or arginine residue. Thus, plasma kallikrein cleaves after a Phe-Arg-sequence in its natural substrate; factor Xa, after a Gly-Arg sequence; and thrombin, after Pro-Arg.

Chloromethyl ketones containing these sequences acquire a relative specificity for the corresponding protease (Table 1). Plasmin, in contrast, generally favours proteolysis at lysyl residues. Advantage may also be taken of differing responses to a D-amino acid residue in P3 or to various side-chain extensions that utilize an additional, possibly unique binding capacity in this region. In the case of serine proteinases, these reagents alkylate the active centre histidine residue.
However, peptidyl chloromethyl ketones also inactivate cysteinyl proteinases and for some in vivo applications may be too reactive. Nevertheless, it has been possible with D-Phe-Pro-ArgCH₂Cl, a rapid inactivator of thrombin, selectively to inactivate this protease within the blood coagulation cascade by intravenous administration of the inhibitor.³

**Peptidyl fluoromethyl ketones**

Some attention has been given to the development of analogous fluoromethyl ketones due to their diminished reactivity compared to chloromethyl ketones and the expectation that a proximity effect produced within the enzyme inhibitor complex would nevertheless promote covalent bond formation.⁴⁻⁷ This has essentially been demonstrated. Peptidyl fluoromethyl ketones are quite rapid in inactivating cysteinyl proteinases whose specificity they satisfy, yet are extremely inert to mercaptoethanol,⁶ a model for the abundant thiols found in biological materials. With respect to serine proteinases, the alkylation rate is slower than that of chloromethyl ketones, chiefly by 1-2 orders of magnitude (Table 2). This loss may well be acceptable if the gain is increased specificity in vivo. However, the fluoromethyl ketones are more difficult to obtain than the chloromethyl ketones.

**Peptidyl diazomethyl ketones**

A different type of a covalent bond forming group is the diazomethyl ketone. Peptides with this C-terminal function are unexpectedly stable. Serine proteinases are generally indifferent, whereas cysteine proteinases are typically very susceptible to members of this reagent class.

\[ \text{R-C-} \text{Cl} = \text{N}=\text{N} \]

\[ \text{O} \]

R = remainder of peptide without carboxyl

It is important at this point to comment on the specificity of cysteine proteinases known to us. Papain, the plant proteinase, is the best studied protease of this family, and, as an increasing number of mammalian proteinases are found to be homologous to it, a papain superfamily is identifiable, including the lysosomal cathepsins B, H, and L⁸ and the cytoplasmic calcium-activated neutral proteinases.⁹,¹⁰ There are other non-homologous cysteine proteinases of microbial origin.

**Table 3. Specificity determinants in the papain family**

<table>
<thead>
<tr>
<th>P²⁻ P¹⁻ P₀⁻ P₁⁻</th>
<th>Cathepsin L, B</th>
</tr>
</thead>
<tbody>
<tr>
<td>-Phe-X-</td>
<td>Cathepsin L, Calpain</td>
</tr>
</tbody>
</table>

Members of the papain family appear to bind substrates by positioning hydrophobic side chains in S₂ and S₃ of the active centre, therefore the nature of the side-chain of the residue whose peptide bond is being hydrolyzed is relatively unimportant. A phenylalanine residue at P₂ has been shown to provide the basis of an effective series of reagents for inactivating cathepsin B and cathepsin L in which the P₁ residue, X, may be basic, hydrophobic, small or large. Although the nature of the amino acid in P₁ does not seem to be specificity determining, it was found that cathepsin L tolerates certain bulky side chains better than cathepsin B in this position and the exploration of this difference led to a considerable enhancement of selectivity. For example, Cbz-Phe-Tyr(O-t-Bu)CHN₂ at 10⁻⁸ M inactivates cathepsin L with a t₁/₂ of 5.7 mins, a rate 2.5 × 10⁴ greater than its action on cathepsin B.¹¹
Cellular studies of the uptake and metabolic effect of peptidyl diazomethyl ketones have shown—either a drop in protein turnover in the case of cathepsin B and L inactivators,\textsuperscript{12,13} a drop in residual intracellular proteolytic activity\textsuperscript{14,15} or the restoration of an enzymatic activity (aryl sulphatase). The latter was observed in the case of a lysosomal storage disease involving an aberrant but still functional aryl sulphatase that was destroyed by catheptic proteolysis.\textsuperscript{16}

Similar considerations of specificity apply to the calcium-activated neutral protease, calpain, found in two forms in the cytoplasm. This proteinase is attracted to hydrophobic amino acid side chains that bind in the S2 and S3 subsites of the substrate-binding region of the active centre, as found by Sasaki \textit{et al}.\textsuperscript{17} from examination of a variety of peptide substrates. A Leu-Leu- sequence is particularly favoured. This led to the synthesis of small peptide inactivators for calpain, such as Leu-Leu-TyrCH\textsubscript{2}Cl\textsuperscript{18} and Cbz-Leu-LeuMetCH\textsubscript{2}N\textsubscript{2},\textsuperscript{19} which rapidly inactivate calpain \textit{in vitro}. Because cathepsin L also has affinity for similar residues in P\textsubscript{2} and P\textsubscript{3}, these inhibitors also inactivate that protease.\textsuperscript{19} However, cathepsin L inactivators containing a phenylalanine in P\textsubscript{2} have no effect on calpain; therefore, ambiguity can be resolved with the use of two inhibitors at the present time. It may be that further work will provide a specific calpain inactivator. It can be expected that such an inhibitor would have wide application in cellular studies on the role of this protease.

\textbf{Peptidylmethyl sulphonium salts}

\[
R-C\textsubscript{6}CH\textsubscript{2}S\textsuperscript{+}(CH\textsubscript{3})\textsubscript{2}
\]

\(R = \) remainder of peptide without carboxyl

A third class of affinity-labelling inhibitors was modelled on S-adenosyl methionine as a biological alkylating agent.\textsuperscript{20} Peptidylmethyl sulphonium salts are particularly effective for inactivating cysteine proteinases and have the advantage of being accessible with amino acid side chains such as arginine whose incorporation into other inhibitor types is chemically difficult.\textsuperscript{21,22}

In connection with protein trafficking, it may be of interest that inhibitors of this type have been applied to the study of prohormone processing. Rat proinsulin is converted to insulin by two endo-proteolytic steps and subsequent trimming. The activities have been separated from insulin secretory granules of rat insulinoma tissue by Hutton and his colleagues\textsuperscript{23} as Ca\textsuperscript{++}-dependent proteases with a low pH-optimum. One of the proteolytic splits follows a Lys-Arg sequence and the other an Arg-Arg- sequence. The activity responsible for the post Arg-Arg cleavage is highly sensitive to inactivation by Ala-Arg-ArgCH\textsubscript{2}S\textsuperscript{+} (CH\textsubscript{3})\textsubscript{2}, but considerably less (orders of magnitude) to Ala-Lys-ArgCH\textsubscript{2}S\textsuperscript{+} (CH\textsubscript{3})\textsubscript{2}. On the other hand, this reagent is more effective in inactivating the enzyme cleaving after Lys-Arg, as expected. The nature of the proteases involved is not yet certain, although this sensitivity is characteristic of cysteine proteases perhaps other types have not yet been adequately examined.

\textbf{Summary}

We have described several classes of peptide derivatives that act as affinity labelling inactivators of serine and cysteine proteinases. Some of these have been shown to enter cells and inactivate the target protease as demonstrated by protein chemistry. In other cases cellular processes have been blocked but the protease involved has not yet been identified.
Extension of these methods can be expected to shed further light on protein metabolism.

References


Table 1. Inhibitor selectivity is a function of sequence (rates of inactivation $10^{-3}$ ×
\[k_{\text{app}}/[I] \text{ (M}^{-1} \text{ sec}^{-1})\]

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Plasma kallikrein</th>
<th>Factor Xa</th>
<th>Thrombin</th>
<th>Plasmin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pro-Phe-ArgCH₂Cl</td>
<td>23.3</td>
<td>0.22</td>
<td>0.02</td>
<td>0.032</td>
</tr>
<tr>
<td>Ile-Glu-Gly-ArgCH₂Cl</td>
<td>4.8</td>
<td>32.0</td>
<td>0.5</td>
<td>0.075</td>
</tr>
<tr>
<td>D-Phe-Pro-ArgCH₂Cl</td>
<td>8.0</td>
<td>4.5</td>
<td>11500.0</td>
<td>0.67</td>
</tr>
<tr>
<td>D-Lys(Bz)-Phe-LysCH₂Cl</td>
<td>0.005</td>
<td>0.23</td>
<td>47.0</td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Comparisons of peptidyl chloromethyl and fluoromethyl ketones

| pH | Cbz-Phe-PheCH₂Cl (m M) | 0.23 | 0.21 | 5   |
|    | Cbz-Phe-PheCH₂F (m M)  | 0.14 | 0.055|
| Cbz-Phe-PheCH₂Cl            | 5.4 | 8.0  | 0.025| 0.115| 7   |
| Cbz-Phe-PheCH₂F             | 5.4 | 0.25 | 0.0015|
| Ala-Phe-LysCH₂Cl            | 7.0 | 0.83 | 0.003| 6   |
| Ala-Phe-LysCH₂F             | 7.0 | 5.0  | 0.00042|
Defining apical sorting in epithelial cells

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References

One of the challenges of contemporary biology is to unravel how the molecular composition of the different cellular compartments is generated and maintained during the cell cycle. In animal cells most of the efforts have been directed towards the study of how newly synthesized proteins are transported to their correct cellular destinations, whereas the lipids, which make up the framework of the membranes in the cell, have been given much less attention.

This paper will focus on a working hypothesis for the generation and maintenance of the different protein and lipid compositions of the two cell surface domains in the polarized cells of simple epithelia. The epithelia lining the body cavities are composed of a single layer of polarized cells. The apical plasma membrane domains of the cells line the lumen of the cavity and the basolateral cell surface faces the underlying extracellular matrix and the blood supply.1,2 Each cell in the layer is linked to its neighbours by intercellular junctions, including the tight junctions that form the permeability barrier between the cells.3 The tight junctions also define the boundary between the apical and the basolateral membrane domains. The sorting of newly synthesized surface glycoproteins defined for the two plasma membrane domains has been localized to the trans-Golgi network, the exit compartment of the Golgi complex.2,4,5 The sorting of newly synthesized lipids enroute for the epithelial cell surface also takes place intracellularly in the Golgi complex,6 raising the possibility that protein and lipid sorting are directly connected to each other. Our working hypothesis is that the transport machinery in the trans-Golgi sorts lipids and proteins into common carrier vesicles for delivery to the correct cell surface domain.

A number of studies indicate that the apical plasma membrane domain is enriched in glycosphinngolipids whereas the basolateral domain has a correspondingly higher phosphatidylcholine content. The distribution of other phospholipids (such as phosphatidylserine and phosphatidylethanolamine), as well as of cholesterol, is similar in the two domains. The asymmetric distribution of lipids in the two surface domains appears to be restricted to the exoplasmic leaflet,2 where their intermixing is prevented by the tight junctions.7,8 The lipids in the cytoplasmic leaflet seem to diffuse freely between the two domains. Therefore, it follows that glycosphinngolipids are exposed on the external surface of the apical membrane, whereas phosphatidylcholine would be exposed on the surface facing the basolateral milieu. The lipids common to both domains such as phosphatidylserine and phosphatidylethanolamine are primarily in the cytoplasmic leaflet. This predicted topology is consistent with the available data on lipid asymmetry in plasma membrane, choline-containing lipids and glycosphinngolipids being generally exoplasmic and amino-containing phospholipids being cytoplasmic.9-13
Apical membranes may have such an unusually high content of glycolipids due to the stabilizing and protective function of these lipids. The apical membranes face the hazards of the external environment. In some epithelial cell types such as those lining the gall bladder and the bile duct, the apical surfaces even have to withstand solubilizing concentrations of the bile salt detergents. Glycosphingolipids are uniquely suited for a protective function because they can form intermolecular hydrogen bonds between the glycosyl head groups, the amide and hydroxyls of the sphingosine base and of the hydroxy fatty acid. To a lesser extent sphingomyelin also has this capacity to associate by intermolecular hydrogen bonds due to its ceramide constituent. This extensive intermolecular hydrogen bonding capacity is a characteristic feature that distinguishes sphingolipids from the major lipid family in animal cells, the glycerolipids. These cannot form interlipid hydrogen bonds between their diglyceride moieties. The ester and ether groups can function only as hydrogen bond acceptors, not as donors.

The sorting event in the trans-Golgi network need not be specific in both the apical and the basolateral directions. It is possible that only one direction is mediated by specific recognition of molecules to be transported (the signal-mediated pathway) and that the other route includes molecules in transit without specific signal recognition (the default pathway). For apical and basolateral membrane proteins, no conclusive answer is available yet, although several attempts have been made to localize the protein signals mediating sorting. One clue might be the tight exclusion of basolateral proteins from the apical side in MDCK cells, whereas the converse is not true. A small fraction of apical proteins are "missorted" to the basolateral side, possibly because the basolateral route operates by default. Biosynthetic protein transport from the Golgi to the fibroblast cell surface has been postulated to be a default pathway. The basolateral route could be the fibroblast homologue. Sorting in the apical direction would be signal mediated, and be specific for simple epithelia. In more complicated epithelial tissues such as liver, the situation is different. Each hepatocyte has several apical poles lining the bile canaliculi. Bartles et al. have shown that apical proteins are not sorted in the trans-Golgi network. There seems to be no apical route from the Golgi complex to the apical membrane in hepatocytes. Instead, apical proteins are delivered to the basolateral membrane, from where they are sorted to the apical side. The simplest interpretation of these findings is that the basolateral transport vesicles (the postulated default pathway) carry both apical and basolateral proteins. The apical sorting machinery seems to be lacking from the trans-Golgi network in hepatocytes.

In MDCK cells, specificity in the apical direction might be aided by glyco-sphingolipid-protein interactions. Sphingolipid clustering in the luminal (exoplasmic) leaflet of the trans-Golgi network is postulated to form the budding site for an apical membrane vesicle. The self-association could be mediated by interlipid hydrogen bonding. This asymmetric sphingolipid microdomain is assumed to be the starting point for inclusion of associating apical proteins that bind directly to the glycosphingolipids or indirectly via interactions to a trans membrane sorting protein. Such a sorting protein should bind to both glycosphingolipids and to the apical proteins. Moreover, this protein is assumed to have another function. Its cytosolic domain interacts with a cytosolic protein coat to induce the curvature leading to vesiculation. According to this model, exclusion of glycerolipids in the exoplasmic leaflet could result from their inability to form interlipid hydrogen bonds with the sphingolipids. The basolateral transport vesicles are predicted to form from membrane regions depleted of apical components and these should, therefore, be enriched in phosphatidylcholine as a consequence of lipid asymmetry. The asymmetry of the lipids facilitates the lateral separation of the apical and the basolateral precursor domains in the trans-Golgi network. Sorting would, according to this view, be the formation of microdomains mimicking the properties of the membranes of their destination. This hypothesis makes several predictions that can be tested. First, there should be specific interactions between glycosphingolipids and apical proteins or between glycosphingolipids and
the putative bridging protein. Second, for exclusion of phosphatidylcholine to occur, the luminal leaflet of the membrane segment that forms the apical transport vesicle has to be almost covered by sphingolipids. The sphingo- to phospholipid ratio of the apical transport vesicles would depend on the size of the vesicle; the larger the vesicle, the nearer the ratio between the surface areas of the luminal and the cytoplasmic leaflets will be to one. Furthermore, if sphingolipid clustering in the trans-Golgi network were a prerequisite for apical delivery, then presumably sphingolipid recycling between the apical membrane and the trans-Golgi would be necessary to replenish the sphingolipids lost in 4 h vesiculation event. Also, the putative sorting protein has to cycle between the apical membrane and the TGN to be able to perform sorting. Sorting proceeds for hours in virus-infected MDCK cells which synthesize only virus proteins and not host proteins.

References


Receptor-mediated endocytosis is studied extensively in liver parenchymal cells. At the sinusoidal, i.e., basolateral cell surface, different receptors and their ligands are trapped in coated pits that pinch-off from the plasma membrane. The vesicles formed lose their clathrin coat and coalesce into a system of anastomosing tubules and connected vesicles, the endocytotic or endosomal compartment. We described this tubulo-vesicular system in relation to the distribution of a receptor that is unique for liver cells, the asialoglycoprotein receptor (ASGPR). This receptor binds galactose terminal glycoproteins, which after internalization are mainly destined for lysosomal degradation. This fate awaits many exogenous products that are taken up by adsorptive endocytosis. Endocytotic products accumulate in endosomal vacuoles. While the vacuoles grow larger, they show increasing numbers of internal vesicles. The vacuoles remain connected to tubular elements. In well-differentiated hepatic parenchymal cells, the endosomal tubules have predominantly a peripheral location and are particularly abundant in the basolateral cell corners. It is here that small endosomal vacuoles seem to arise. In less polarized cells, such as the cultured hepatoma cell lines Hep-G2 (human) and H4S (rat), the endosomal elements occupy less characteristic positions in the cells. In these cells the endosomal tubules and those of the trans-Golgi reticulum (TGR) are easily mixed up when observed by electron microscopy (EM). Therefore, in immunocytochemical studies endocytosed markers like cationized ferritin or specific ligands, tagged to electron-dense markers for EM detection, are required to distinguish endosomes from TGR. The elements of the TGR, on the other hand, can be recognized by marking endogenous secretory proteins, such as albumin in liver cells.

Significant differences exist in the molecular composition of the plasma membrane and of the lysosomal membrane. Intensive membrane remodelling along the prelysosomal route seems the obvious way to maintain this difference in spite of a conspicuous membrane flow resulting from the vectorial transport of exogenous material to lysosomes. The intermediate composition of endosomes that we observed for several membrane proteins illustrates that such membrane remodelling indeed takes place (Table 1). In several studies we investigated these changes in the endosomal compartment by observing individual membrane proteins after immunogold labelling in thin cryosections.

Immunocytochemistry showed that the membrane of coated pits is, to some extent, different from the rest of the plasma membrane. Pits were enriched in ligand-receptor complexes. The pits appeared to be non-selective for different receptors. The ASGPR occurred together with the mannose 6-phosphate receptor (MPR), which recognizes the phosphomannosyl residues on lysosomal enzymes. These two receptors also co-localized to coated pits with the receptor...
for polymeric IgA (IgAR). Recently, careful analysis of endosomal elements in cell fractions demonstrated that the transferrin receptor (TFR) system entered Hep-G2 cells together with the ASGPR system. Hence, at least 8 different compounds (4 receptors + 4 ligands) enter the same elements of the endosomes. Of only the ligands of ASGPR and MPR, the main destination is the lysosomal compartment. TFR with ligand and ASGPR recycle to the cell surface. The MPR is also known to be absent from lysosomes. The IgAR with covalently linked ligand is directed to the bile capillary (apical) cell membrane, where it is cleaved from the membrane and secreted. By comparing the distribution of these different molecules in immunodouble-labelled sections, we studied the sites of selective removal of the ASGPR, MPR and the IgAR-ligand complex in liver cells.

Within the peripheral endosomal tubular system we found microdomains enriched in IgAR and ASGPR, indicating a very early sorting of the two receptor systems. This is in agreement with results demonstrating that segregation of endocytosed IgA and ASGPR ligand occurs during the very first minutes after uptake. We observed accumulation of ASGPR ligand in the endosome vacuoles, whereas ASGPR was present mainly in the tubules. Therefore, we called this tubulo-vesicular compartment CURL, i.e., compartment of uncoupling receptor and ligand. Recently, the distribution of ASGPR in endosomes was studied in more detail. It was shown that the larger the endosomal vacuoles were, the less ASGPR was present in their limiting membranes (Figures 1, 2). ASGPR seemed to accumulate in the tubules. It was proposed that the ASGPR and ligand dissociate in the acidic environment inside the endosomal vacuoles and that the receptor molecules then migrate laterally into the tubules. Since receptor negative vacuoles are often seen in continuity with receptor enriched tubules (Figure 2), we propose an efficient block occurs at the junction of tubules and vacuoles that prevents the receptor from moving back into the vacuoles and the ligand from moving in the reverse direction.

Besides the sorting of IgAR and ASGPR in peripheral early endosomes, we studied the intracellular pathways of two other membrane proteins in endosomes, which occurred predominantly in the deeper (late) endosomes, namely the MPR and a 120-kD lysosomal glycoprotein, LGP120. MPR enters the endosomes by coated vesicles together with ASGPR at a concentration that is almost proportional to the concentration of both receptors at the sinusoidal cell surface. Nevertheless, quantitation of the immunolabelling in Hep-G2 cells demonstrated that MPR is 5 times more abundant in endosomes than ASGPR relative to their densities at the plasma membrane (Table 1). Most likely this is due to two phenomena. First, MPR is segregated at a later stage from the endosomes than ASGPR. This fits well with the impression that late endosomal vacuoles (large vacuoles with many internal vesicles) were still labelled for MPR. Second, there may occur an additional supply of more MPR molecules to endosomes via another route than coated pits. A significant pool of cellular MPR is localized in the TGR of Hep G2 and H4S cells (Figure 3). In the TGR the receptor and lysosomal enzymes seem to accumulate in characteristic electron-dense, clathrin-coated buds and vesicles. Similar vesicles are often observed adjacent to endosomal vacuoles. This suggests a direct transport of MPR from TGR to endosomes, by-passing the plasma membrane. In the endosomes, MPR is usually enriched in tubules attached to the vacuoles, which indicates that MPR, like ASGPR, is recaptured from the lysosomal route by lateral migration from the vacuoles into the tubules. This would finally lead to the absence of MPR from lysosomes.

It was therefore of interest to compare the MPR distribution with that of the typical lysosomal membrane protein LGP120, which we found at least as abundant in endosomes as in lysosomes of H4S cells (Table 1). This is in agreement with observations of other cell types. LGP120 occurred in vacuoles, but not in tubules of endosomes. The cell surface was virtually
devoid of LGP120, whereas the Golgi complex and TGR showed less than 5% of the total cellular LGP120 labelling.

We offered H4S cells cationized ferritin (CF) for 5, 10, 30 or 60 min prior to fixation, prepared cryosections and studied the distribution of MPR and LGP120 by immunolocalization. At each time interval we counted the number of CF containing vacuoles, (i.e., endosomal vacuoles and lysosomes), labelled for MPR and LGP120 (Figure 4). The complete data are reported elsewhere.15 Apparently, CF is taken up in a very short living category of vacuoles in which neither MPR nor LGP120 was detectable. Then a rapidly growing fraction of the CF-positive vacuoles became labelled for MPR, which peaks at 10 min. The increase of MPR labelling during the first minutes is in agreement with the above and suggested additional direct transport of this receptor from TGR to endosomes. Furthermore, the main removal of MPR from the endosomal vacuoles seems to take place after 10 min, The ASGPR, on the other hand, has been reported to be segregated from the degradative route within the first few minutes after endocytosis.7 This is consistent with our impression that MPR, in contrast to ASGPR, occurs in relatively late endosomal vacuoles. The LGP120+ fraction of CF-containing vacuoles increases more slowly than those with MPR. Increasing percentages of 50, 80 and 90% of the MPR+ CF vacuoles were also labelled for LGP120 at 10, 30 and 60 min, respectively (not shown in Figure 4). Conversely, the percentage of LGP120+ CF vesicles that labelled also for MPR decreased according to 75, 50 and 30% at 10, 30 and 60 min, respectively. Since MPR-/LGP120+ vesicles can probably be defined as lysosomal structures, the latter figures give an impression of the rate of uptake of CF in lysosomes. After 1 h approximately two thirds of the CF containing structures are lysosomes.

About half of the LGP120 labelling is located in the endosomal vacuoles (Table 1). Only a very little LGP120 is found in the Golgi area, which is consistent with a proposed fast transport of newly synthesized LGP120 to lysosomes.16 It also suggests that the protein does not recycle in circuits in which Golgi elements or TGR participate, as is probably the case for ASGPR and MPR.9,17,18 Therefore, the inflow of LGP120 into endosomes from the Golgi complex or TGR, either via the cell surface or directly by vesicles, is probably less significant than for the other receptor molecules that we described. Instead, the high concentration of LGP120 in the late vacuoles, is likely to be a result of recycling molecules from the lysosomes back into the endosomal vacuoles although there is no direct evidence for such a recycling route. Small irregular membranous structures rich in LGP120 may function as intermediates. They are found to contain approximately 10% of cellular LGP120 labelling. That with time an increasing percentage of CF positive vesicles marked for MPR also contained LGP120 demonstrates that the bulk of LGP120 enters the endosomes at a later stage than MPR. However, some MPR+/LGP120+ vacuoles were reached by CF very soon after uptake. Therefore, it is likely that LGP120 is added gradually along a relatively long stretch of the endocytotic route.

Our observations are summarized in Figure 5. For three receptors and a major lysosomal membrane protein we describe the sites of entry into and segregation from endosomes in hepatocytes. Our observations are in agreement with a gradual transformation of the endosomal membrane from a plasma membrane-like composition immediately after endocytosis into one that resembles more closely the lysosomal membrane.

References


Table 1. Immunogold labelling in cryosections of hepatoma cell lines (Hep-G2 and H4S) for ASGPR, MPR and LGP120 in plasma membrane (PM), endosomes (End) and lysosomes (Lys).

<table>
<thead>
<tr>
<th></th>
<th>HepG2 (PM: 51 (39) 18 (9) 16 (10) 1 (1))</th>
<th>H4S (End: 48 (37) 82 (42) 84 (53) 58 (50))</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASGPR(1)</td>
<td>a 18 (9) b 16 (10) a 1 (1)</td>
<td>a 82 (42) b 84 (53) a 58 (50) b 41 (36)</td>
</tr>
<tr>
<td>MPR(2)</td>
<td>a b a b</td>
<td>a b a b</td>
</tr>
<tr>
<td>MPR</td>
<td>a b</td>
<td>a b</td>
</tr>
<tr>
<td>LGP120</td>
<td>a b</td>
<td>a b</td>
</tr>
</tbody>
</table>

a: relative distribution of gold particles over the three compartments mentioned.

b: percentage of particles of total cellular labelling.
Figures 1 and 2. Endosomal elements of rat liver cells, immunolabelled for asialoglycoprotein receptor (ASGPR). Labelling is most prominent in tubules and small vesicles (Figure 1). Large vesicles (Figure 2), containing internal vesicles, are not labelled, even though they are connected to ASGPR positive tubules (arrowhead).

Figure 3. Mannose 6-phosphate receptor (MPR) immunolabelling of the Golgi area in a Hep-G2 cell. Golgi cisternae (asterisks) are almost devoid of MPR but tubules and dense vesicles (arrows) of the trans-Golgi reticulum (TGR) seem to contain the receptor at relatively high concentrations. The dense vesicles are thought to pinch off from the TGR and transport MPR directly to endosomal vacuoles, an example of which is depicted (inset), double labelled for MPR (9 nm gold particles) and asialoglycoprotein receptor (ASGPR) (6 nm gold particles). The strong ASGPR labelling characterizes this example as an early vacuole.
Figure 4. Percentage of cationized ferritin (CF) containing vacuoles in H4S cells, in which mannose 6-phosphate receptor (MPR) (MPR+), LGP120 (LGP120+) or none of these molecules (MPR-/LGP120-) were detected by immunogold labelling after incubating the cells for various periods with CF (Geuze et al., in press).

Figure 5. Impression of membrane remodelling in the endosomes of liver cells. The arrows indicate at which level inflow and segregation of typical endosomal membrane proteins occur.
Endocytosis by African trypanosomes

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**Transferrin binding**

**Cell association of transferrin at 37 °C**

**Cell association of ⁵⁹Fe-transferrin**

**Transferrin-gold binding and uptake**

**Uptake of bovine serum albumin and transferrin coupled to gold**

**Binding and uptake of horseradish peroxidase (HRP)**

**Uptake of HRP and transferrin-gold**

**Conclusions**

**References**

African trypanosomes are protozoan flagellates that are responsible for extensive disease in humans and domestic animals across a large area of Africa. The success of trypanosomes lie in their ability to avoid the host immune response by varying, both biochemically and antigenically, the glycoprotein coat (variable surface glycoprotein, or VSG) covering the entire cell surface during the course of infection in the mammalian host.¹,²,³

It is believed that the surface glycoproteins of trypanosomes are too antigenically diverse to be useful as a material for a vaccine.⁴ However, other antigenically sensitive sites vulnerable to antibody attack may be revealed as the cell biology of the organism is studied. A process that may serve as a target for antibody attack within trypanosomes is endocytosis.

Trypanosomes, like all living cells, require materials from their environment for continued growth. Many molecules enter cells by endocytosis, either by fluid phase or by absorptive or receptor-mediated endocytosis of selected molecules via clathrin-coated vesicles.⁵ African trypanosomes in the bloodstream of infected animals take up various particulate and soluble substances in vesicles morphologically similar to the clathrin-coated vesicles found in other eukaryotic cells.⁶,⁷,⁸ These vesicles bud only from the membrane of the flagellar pocket, a deep invagination of the plasma membrane where the flagellum leaves the cell, and discharge their contents into an intracellular tubular system.⁶

Iron, essential to all cells, is supplied to mammalian cells primarily by a major serum protein called transferrin, which, when iron loaded, will bind to receptors on the cell surface. The receptor-ligand complexes are internalized by receptor-mediated endocytosis⁹ through clathrin-coated pits and vesicles⁰,¹¹,¹² and are delivered to the endosome compartment of the endocytic pathway.¹¹,¹³,¹⁴ Here, the low pH environment¹⁴,¹⁵,¹⁶ triggers the dissociation of
iron from the transferrin\textsuperscript{17,18} and the iron is transported across the membrane. The iron-free or apotransferrin-receptor complexes recycle to the cell surface, where, at neutral pH, the apotransferrin dissociates from the receptor and the cycle is repeated\textsuperscript{10,11,17,18}.

African trypanosomes also require iron for growth (R. Kaminsky, personal communication). For these cells, however, the mechanisms by which iron is acquired are not understood. We have shown that colloidal gold coupled to diferric transferrin bind to the surface of trypanosomes and are internalized by vesicles via the flagellar pocket\textsuperscript{8}. Uptake of VSG molecules also occurs via the same vesicles.\textsuperscript{8} It has recently been demonstrated that transferrin and low-density lipoproteins enter \textit{Trypanosoma brucei} by receptor mediated endocytosis\textsuperscript{7}. However, little is known about the nature of the parasite receptors, or the fate of substances internalized by trypanosomes.

This report will investigate the fate of various endocytosed substances in African trypanosomes.

All experiments were carried out on \textit{T. brucei} populations isolated from infected rat blood, as previously described\textsuperscript{19}.

**Transferrin binding**

Trypanosomes were incubated with \textsuperscript{125}I diferric transferrin on ice. At the indicated times, free ligand was washed away and the cell associated radioactivity was determined. Binding was slow and took up to 3 h to reach a plateau. The binding was saturable and was competitively inhibited by excess unlabelled diferric transferrin. Binding of \textsuperscript{125}I apotransferrin was found to be only 12\% efficient when compared with diferric transferrin binding.

**Cell association of transferrin at 37 °C**

\textit{Trypanosoma brucei} incubated with either \textsuperscript{125}I diferric or \textsuperscript{125}I apotransferrin were placed on ice and, at increasing time intervals, washed with cold medium to remove unbound label and transferred to fresh tubes to determine the cell associated radioactivity.

At 37 °C diferric transferrin rapidly associated with the cell and reached a maximum of cell-associated activity at 10 min after the onset of incubation at 37 °C. Subsequently, the cell associated activity declined, suggesting that the protein was released from the cells.

**Cell association of \textsuperscript{59}Fe-transferrin**

To determine whether the iron associated with diferric transferrin remained cell-associated, trypanosomes were incubated in \textsuperscript{59}Fe-transferrin. At increasing times the cells were washed and the cell-associated radioactivity was determined. A steady, linear accumulation of cell-associated radioactivity was seen over a 90 min period.

In the presence of ammonium chloride, the accumulation of radioactivity was initially inhibited, but within 30 min the rate of ferric \textsuperscript{59}Fe uptake was equivalent to that in the untreated samples. The results suggest that iron accumulates in trypanosomes after dissociating from transferrin in an acidic compartment. In the conditions used for these experiments, the cells rapidly acidify the medium and as a consequence NH\textsubscript{3} is protonated, becomes less permanent to membranes and loses its ability to neutralize intracellular acidic compartments.

**Transferrin-gold binding and uptake**
Trypanosomes were incubated on ice in medium containing transferrin coupled to colloidal gold particles. By electron microscopy, the gold particles were seen to bind to the surface of the trypanosomes. Occasionally gold particles were found in flagellar pockets, but most pockets did not contain the marker.

When trypanosomes were warmed to 37°C in the presence of transferrin-gold, the gold particles were found in the flagellar pocket, in pits on the flagellar pocket membrane and in intracellular vesicles in close proximity to the flagellar pocket. After a 5-min incubation at 37°C, the gold markers were found in intracellular structures with tubular profiles and large vesicular and lysosome-like structures.

If trypanosomes, incubated in transferrin-gold, were reacted with a silver intensification solution and viewed by light microscopy, a dark reaction product revealing the colloidal gold was present in the region between the nucleus and the flagellar pocket, situated at the posterior end of the cell. Reaction product was seldom found in the anterior part of the cell.

**Uptake of bovine serum albumin and transferrin coupled to gold**

When trypanosomes were incubated in medium containing transferrin coupled to colloidal gold of one particle size and bovine serum albumin (BSA) coupled to gold of a different particle size, both markers bound to the cell surface. Both markers entered the cell via the flagellar pocket and in the same intracellular vesicles. No segregation of the two markers was seen in any of the endocytotic organelles except that some vesicular structures contained only the smaller gold particles, regardless of the coupled protein.

**Binding and uptake of horseradish peroxidase (HRP)**

Trypanosomes incubated on ice in medium containing HRP were seen to have horseradish peroxidase (HRP) bound to the cell surface, after visualization with diamino-benzidene. The flagellar pockets did not contain HRP. Upon warming to 37°C, the HRP entered the cells via the flagellar pocket and after 5 min was found in many intracellular structures. Often tubular structures near to the Golgi apparatus were filled with HRP, but there was no consistent association of HRP with the Golgi.

Serial sections through trypanosomes containing endocytosed HRP produced a pseudo three-dimensional view of the endocytotic organelles and revealed them to be a complex tubulo-vesicular network.

**Uptake of HRP and transferrin-gold**

When transferrin-gold was included in the incubation medium with HRP, the two markers co-localized in the flagellar pocket and in many intracellular organelles. Whereas the transferrin-gold always co-localized with the HRP, the HRP was found in vesicles close to the flagellar pocket and in many tubular and vesicular structures that did not contain transferrin-gold. Many of these HRP-containing structures were connected to structures that contained the colloidal gold marker.

A fractional volume analysis revealed that transferrin-gold containing structures constituted 2% of the total cell volume, whereas the HRP-containing structures constituted 5% of the total cell volume.

**Conclusions**

It appears that trypanosomes obtain iron from the blood that surrounds them in a way similar to that used by the mammalian hosts. Receptors on the surface of trypanosomes specifically
bind diferric transferrin, and the receptor-ligand complexes enter the cell, where they meet an acidic environment that causes the iron to dissociate from the transferrin. The eventual fate of the internalized transferrin has not yet been determined.

Morphologically, using transferrin coupled to colloidal gold, it seems that trypanosomes internalize transferrin through coated vesicles into a tubulo-vesicular compartment similar to the endosome compartment of mammalian cells. The precise morphological details of the endocytotic uptake of transferrin must await analysis using monomeric protein, as it is clear the proteins coupled to colloidal gold may be directed to sites other than those reached by uncoupled proteins.

It is clear that the organelles involved with endocytosis are morphologically complex structures when visualized by HRP and that colloidal gold particles enter only parts of the endocytic pathway. Endocytosis by African trypanosomes is important for nutrient uptake and possibly for processing of intracellular VSG. An understanding of the complex mechanisms involved may reveal new ways to control trypanosomiasis.

References

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