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CHARACTERIZATION OF INVARIANT MEMBRANE PROTEINS OF *TRYPANOSOMA (DUTTONELLA) VIVAX*.

by

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Department of Microbiology and Immunology

Submitted in partial fulfilment of the requirements
for the degree of Doctor of Philosophy

Faculty of Graduate Studies
University of Western Ontario
London, Ontario
August, 1992

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ABSTRACT

Monoclonal antibodies (mAbs), raised against whole, fixed, uncoated, culture forms of *Trypanosoma (Duttonella) vivax*, were used to identify two invariant membrane proteins of this protozoan parasite. Since non-variant membrane proteins of the cell surface, flagellar pocket and endocytic pathway are potential targets for the control of trypanosomiasis of livestock by immunization, the identification and characterization of invariant membrane proteins is a necessary preliminary step.

A 65 kDa invariant membrane glycoprotein (gp65), identified using mAb 4E1, was the main focus of this study. Immunolocalization studies using the monoclonal antibody (mAb 4E1) for immunofluorescence staining and immunoelectron microscopy, demonstrated that the 65 kDa antigen was associated with tubulo-vesicular profiles in the posterior region of the bloodstream form parasite. Endocytosis and co-localization experiments revealed that gp65 was associated with an endocytic compartment of *T. vivax* which is morphologically and temporally similar to the endosomal system of mammalian cells. Double labelling experiments using the mAb and a polyclonal anti-variant surface glycoprotein antibody (RαVSG) to simultaneously localize both gp65 and intracellular VSG, demonstrated that there was little overlap in the distribution of these antigens. Thus, gp65 is associated with tubules and vesicles that are involved in endocytosis but which appear to be distinct from VSG processing pathways in the cell.

A 35 kDa *T. vivax* antigen was shown in immunolocalization studies to be associated primarily with the surface of bloodstream forms of the parasite. Although *T. vivax* 3'-nucleotidase activity, a surface membrane enzyme in other trypanosomatids, migrated at 35 kDa on SDS-PAGE gels, it is doubtful that the 35 kDa antigen identified with the monoclonal antibody (mAb 4B11) is specific for the *T. vivax* 3'-nucleotidase since the two proteins exhibited different capacities to bind to immobilized Concanavalin A.

Both *T. vivax* invariant antigens have potential as targets for disease control based on their location in the cell and thus merit further study to this end. In addition, gp65 is the first putative marker for an endosomal compartment of trypanosomes and has potential for use in the further study of endocytosis in African trypanosomes, a process upon which these parasites are dependent upon for survival.

This thesis is dedicated to my grandmother, Kathleen B. Edwards, who was denied an opportunity to pursue a career in science and to my goddaughter, Leanne Greenwood, who has the world before her.

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I would also like to thank Paul Webster, not only for convincing me that immunocytochemistry using ultrathin cryosections was an important technique and dedicated many hours to patient instruction, but who had faith in my abilities, and through much interaction added new perspective to my life.

There are many people at ILRAD that I would like to thank. Clive Wells expanded my knowledge of immunocytochemistry and contributed much to this thesis, both in terms of skill and critical evaluation. Sammy Kemei and Stephen Wasike were a tremendous help in the often tricky task of growing and isolating *T. vivax.* Ged Lamb taught me how to make monoclonal antibodies which were crucial for the success of this project. Terry Pearson, during his 18 month visit to ILRAD, contributed expert technical advice, enthusiasm, and daily antecdotes which were greatly appreciated. The people in the small animal unit, central core, photography and graphic arts provided services that invaluable in making life easier and more efficient.

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CHAPTER 1

Literature Review

1.1 Distribution of *Trypanosoma vivax*

Trypanosoma (Duttonella) vivax, one of the etiological agents of trypanosomiasis of livestock, has both epidemiological and economic importance in Africa, South America and the Carribbean. In Africa, *T. congolense*, and to a lesser extent *T. brucei*, are also significant, but their distribution is restricted to that of their obligate insect vector, the tsetse fly (*Glossina* species) whose range lies between 15°N and 25°S (Hoare, 1957). Although *T. vivax* is transmitted by the tsetse fly in Africa, it can also be transmitted by other biting insects (Hoare, 1972), so that it now has a larger distribution in Africa and has spread to South America possibly via export of infected cattle from the West African coast (discussed in Hoare, 1947). In Africa, the epidemiological importance of mechanical transmission is controversial. *T. vivax* infections were prevalent in areas of Ethiopia where there was an apparent absence of tsetse (Roeder et al., 1984), and were still seen in an area of Chad after tsetse eradication (Taze and Gruvel, 1978). Thus this mode of transmission is, at least, a contributing factor to the epidemiology of the disease in Africa. In South America, no definitive insect vectors have been identified, but there are many candidates including tabanids, triatomine bugs, ticks and mosquitoes. In addition, it would appear that the mechanically transmitted *T. vivax* isolates from South America have lost the ability to undergo cyclical development in the tsetse fly (Roubaud et al., 1938; Dirie et al., 1992).

1.2 Morphology

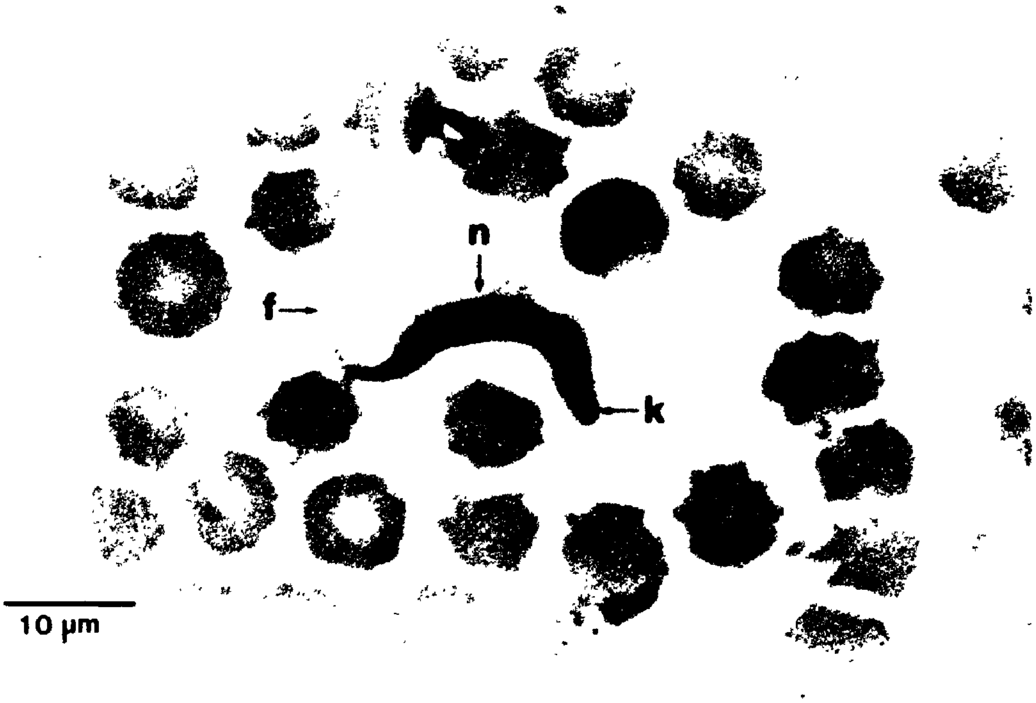
T. vivax is a kinetoplastid protozoan which exists as extracellular trypomastigotes and epimastigotes with a single flagellum. The flagellum originates posteriorly in trypomastigotes and from a juxtanuclear region in epimastigotes. It extends forward, attached to the body of the trypanosome by an undulating membrane except at the anterior end where the flagellum is free, extending beyond the end of the cell (Fig. 1.1). The body of the trypomastigotes posterior to the nucleus is swollen and the anterior portion narrows rapidly (Bruce et al., 1910a). Bloodstream form trypomastigotes are 20-26 μm in length (Hoare and Broom, 1938; 1939) and have a distinctive, rapid, vibrational movement when observed under the light microscope in a wet blood film (Bruce et al., 1910a). The kinetoplast of *T. vivax* is also a diagnostic feature, being large, terminally placed and cup-shaped in electron micrographs of thin sections of the parasite (Vickerman and Evans, 1974). *T. vivax* was long thought to be monomorphic but pleomorphic forms can be found in the blood of an infected animal. "Late" forms, which have been noted in both rodent and cattle infections, occur when parasitemias are quite high. They are longer and more granular than the bloodstream forms found earlier in infection, and have large vacuoles and a sub-terminally placed kinetoplast (Shapiro et al., 1984; Mahan, 1984; Dirie et al., 1986;

Figure 1.1

Bloodstream form *T. vivax*

A Giemsa-stained blood smear taken from an infected cow. The posteriorly placed kinetoplast (k), the nucleus (n) and single flagellum (f) are visible in this light micrograph. Scale bar = 10 µm.

Bloodstream form T. vivax



Gathuo et al., 1987; Fish et al., 1987; Gumm, 1991). These forms swarm around blood cells or amorphous matter to which they adhere via the tip of their flagella (Fish et al., 1987; Gumm, 1991). It is likely that these forms are already partially adapted for attachment and subsequent development in the tsetse fly. Indeed, it is best to use a population which consists predominantly of late forms when seeding cultures to generate other insect stages of *T. vivax* (Gumm, 1991). These late forms will easily stick to the plastic of the tissue culture flask which probably simulates the situation of adherence to the proboscis of the tsetse fly.

1.3 Life Cycle in Tsetse

The best studied animal trypanosomes, *T. brucei*, *T. congolense* and *T. vivax*, have characteristic cycles of development in tsetse and the location of trypanosomes in the fly is often used as a diagnostic feature. A generalized life cycle for the three parasites is illustrated in Fig. 1.2. To summarize, bloodstream form trypanosomes are ingested by the tsetse fly while feeding on an infected animal. In the tsetse fly, *T. brucei* and *T. congolense* bloodstream form parasites reach the midgut and differentiate into elongated, uncoated procyclic forms. Procyclics differentiate to form attached epimastigotes, (*T. congolense* attaches to the wall of the proboscis of the fly whereas *T. brucei* attaches within the salivary glands), and will further differentiate at these sites into free swimming metacyclic forms which are infective for the mammalian host.

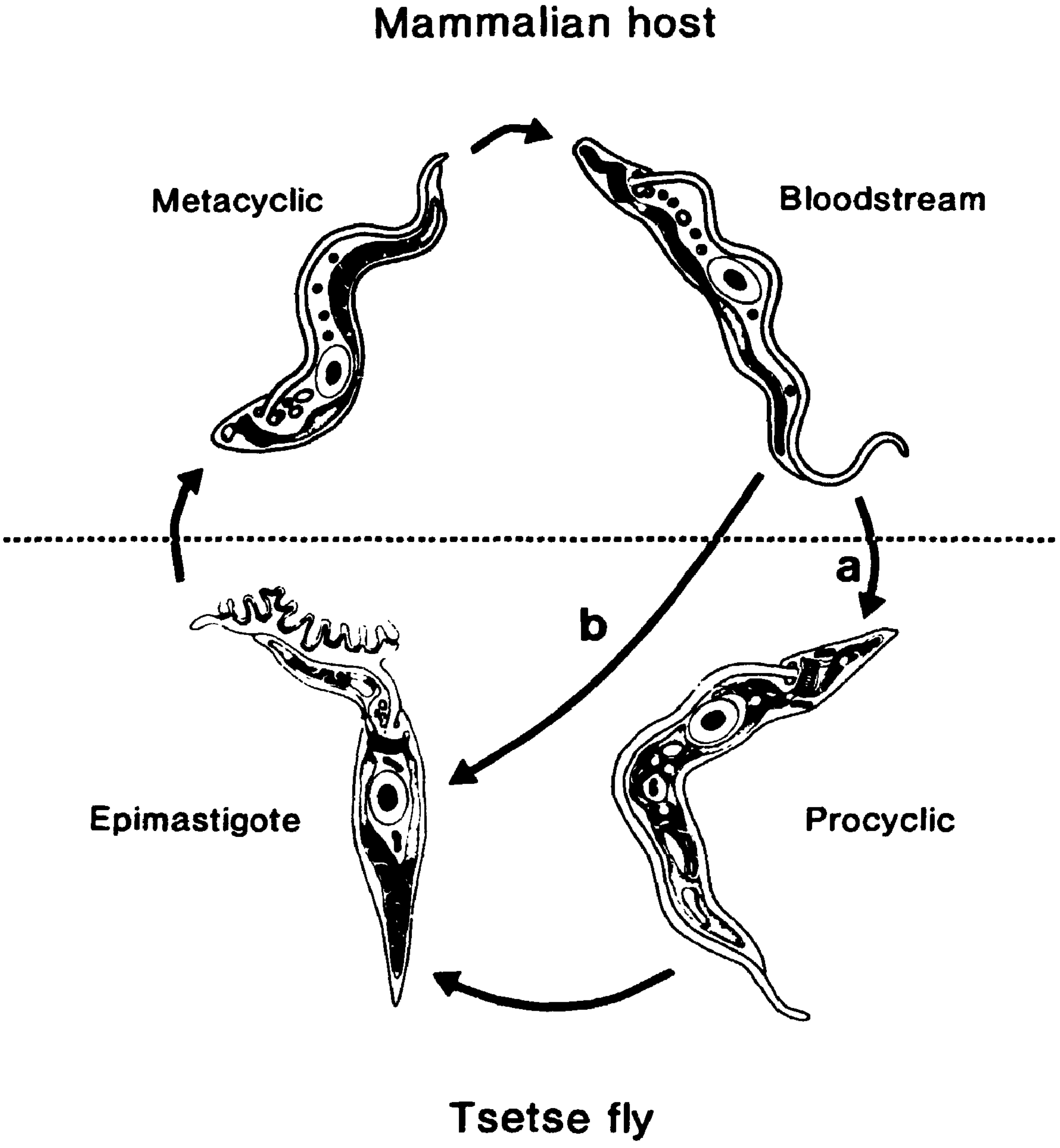
T. vivax has a simplified life cycle in tsetse, in that it is largely confined to the proboscis (Bruce, 1910b; Jeffries, 1984; Jeffries et al., 1987; Moloo and Gray, 1989). Furthermore, there is no life cycle stage of *T. vivax* corresponding to procyclic forms, thus bloodstream forms differentiate to epimastigote forms directly (see Fig. 1.2). Epimastigotes, whose kinetoplast is placed either juxtanuclear or on the anterior side of the nucleus, actively divide to form bundles which are still attached to the wall of the labrum, colonizing the entire length (Vickerman, 1973; Jeffries, 1984). Infective metacyclics, which are adapted to life in the mammalian host due to the reacquisition of the surface coat (Gardiner et al., 1986a), are free swimming and begin to appear in the hypopharynx after 5-13 days of development in the fly, depending on the temperature (Desowitz and Fairburn, 1955). Metacyclics are released into the skin of the host when an infected tsetse fly takes a bloodmeal and they differentiate into bloodstream forms which are then released into the circulation and establish infection. For *T. brucei* and *T. congolense*, differentiation to bloodstream forms from metacyclics in the skin takes several days, thus metacyclic and skin forms contribute to immunity in infections with these parasites. In contrast, metacyclics do not routinely provoke immunity in *T. vivax* infections (see section 1.6).

During the differentiation of bloodstream form trypomastigotes to the insect vector stage of the parasite, the dense surface coat of variant surface glycoprotein (VSG) molecules (see section 1.5.4) is lost (Barry and Vickerman, 1979). It is replaced with a stage-specific glycoprotein, procyclin (Richardson et al., 1988), after a rapid increase in procyclin-specific mRNA synthesis (Roditi, et al., 1989). Procyclin

Figure 1.2

Life cycle of African trypanosomes.

African trypanosomes undergo cyclical differentiation within the mammalian host and the tsetse fly vector. The life cycle of *T. brucei* and *T. congolense* include a defined insect vector form called the procyclic form (pathway a) whereas *T. vivax* bloodstream forms differentiate to epimastigotes directly (pathway b).


Mammalian host
Metacyclic
Bloodstream
a
b
Epimastigote
Procyclic
Tsetse fly

is an acidic glycoprotein molecule with many repeated glutamic acid-proline residues in the amino acid sequence (Roditi et al., 1987), and is thought to have a glycolipid anchor (Clayton and Mowatt, 1989) which differs from the VSG membrane anchor (Ferguson et al., 1988). The procyclin molecules from *T. brucei* are conserved within this species but differ immunochemically and biochemically from *T. congolense* procyclin (Roditi and Pearson, 1990). The presence of a procyclin-like molecule on the surface of *T. vivax* insect forms has not been established, but Southern blot analysis using the *T. brucei* procyclin gene to probe *T. vivax* DNA indicate that sequences related to procyclin are present in *T. vivax* (Roditi et al., 1987). This question can now be more easily addressed due to the recent development of axenic culture systems in which all of the life cycle stages of *T. vivax* can be generated and maintained (Gumm, 1991). When the bloodstream form of *T. vivax* is used to seed cultures and incubated at 27°C, a non-infective, non-dividing form which lacks the dense surface coat is seen after 3 days during the differentiation of bloodstream forms to epimastigote forms.

1.4 The Disease

1.4.1. The Chancre

The first obvious sign of human trypanosomiasis is an inflammatory skin lesion, termed the chancre, at the site of the tsetse fly bite (Roberts et al., 1969). However, the importance of the chancre in animal trypanosomiasis has been questioned since they are rarely observed in natural infections although they can be elicited in experimental infections (Roberts et al., 1969; Luckins and Gray, 1978). The chancre appears several days after *T. brucei* infection and from as early as 12-24 hours to several days before parasites can be detected in the circulation (Emery and Moloo, 1981). The inflammatory response is characterized by edema and an early infiltration of polymorphonuclear leucocytes. Later, many lymphocytes, macrophages and plasma cells are found concentrated in the area. Small numbers of parasites have been found in the lesion on the eighth day after infection (Akol and Murray, 1982) and in the deeper dermal layers 6-15 days after infection (Emery and Moloo, 1981).

In the large chancres produced by *T. congolense* infection, there is extensive multiplication of metacyclics in the skin (Luckins and Gray, 1978) which can be detected up to 2 weeks after infection. When chancres are produced in experimental *T. vivax* infections they tend to be smaller which may be due to the fact that only a few metacyclics are extruded upon tsetse feeding (Otieno and Darji, 1979; Gardiner et al., 1986a). However, chancres are not always apparent (see Gardiner, 1989) and the metacyclics do not appear to multiply to the same extent or remain at the skin site for as long as *T. congolense* metacyclics (Emery et al., 1980). In both *T. brucei* and *T. congolense* infections, the appearance of chancres is accompanied by an increase of the draining lymph node to 2-3 times its normal size. In *T. vivax* infections, swelling of the draining lymph node and parasites in the lymph can be detected before any changes were detected at the bite site (Akol and Murray, 1983).

1.4.2. The Parasitemia

After trypanosomes enter the circulation of their hosts, they divide by binary fission with a doubling time of approximately six hours. Peak parasitemias in livestock do not usually exceed 10^6 trypanosomes/ml whereas they can reach levels as high as 10^9 trypanosomes/ml in immunocompromised laboratory rodents. During the course of infection, parasitemias rise and fall, with the peaks of parasitemia becoming less pronounced as the infection progresses (Gray, 1965) The general symptoms accompanying the parasitemia are anemia, fever, weight loss, decreased milk yield and decrease in fertility (Betancourt, 1978). The major pathological changes that occur are in the lymph nodes and spleen which undergo generalized enlargement and in the pituitary gland, testicles and heart which become inflamed (Masake, 1980).

The major cause of death in livestock due to trypanosomiasis is through persistent anemia and congestive heart failure due to myocardial damage (Murray et al., 1977). The initial wave of parasitemia is accompanied by anemia, leucopenia, and thrombocytopenia (Maxie et al., 1976; Esievo and Saror, 1983). Most of the red cell destruction occurs in the spleen in mildly anemic animals and in the liver in severely anemic animals (Anosa and Isoun, 1980). In addition, there appears to be an absence of regenerative forms of red cells, which suggests an interference with erythropoiesis Immunoglobulins and a complement component, C3, have been found to adhere to the erythrocytes of cattle infected with *T. vivax* (Maxie et al., 1976; Facer et al., 1982) IgM and IgG eluted from red cells have been shown to be reactive to soluble *T. vivax* antigens (Facer et al., 1982). Therefore, either antigen-antibody complexes are binding to the surface of red blood cells during infection or the red cells become coated with trypanosome proteins to which specific antibody subsequently binds. The net effect of either of these mechanisms is a rapid clearing of the opsonized red cells by phagocytosis, or complement lysis, which contribute to the anemic state.

Some stocks of *T. vivax*, particularly in East Africa, can cause an acute disease in cattle which is accompanied by a hemorrahagic syndrome (Mwongela et al., 1981; Wellde et al., 1983). The disease is characterized by a very high initial parasitemia (10^5-10^6 parasites/ml), fever, profound anemia, and hemorrhages in the viscera and mucosal surfaces especially in the gastrointestinal tract. The acuteness of the disease in cattle often results in death or abortions before diagnosis and treatment (Mwongela et al., 1981).

1.4.3. Chemotherapy

There are few trypanocidal drugs that are widely used to combat animal trypanosomiasis. The most commonly used drugs for *T. vivax* infection are: homidium chloride, homidium bromide, isometamidium chloride, quinapyramine dimethylsulphate, quinapyramine dimethylsulphate:chloride 3:2 (w/w), and diminazene aceturate. Quinapyramine chloride and isometamidium have prophylactic activity whereas the others are only effective as therapeutic agents.

After drug treatment, relapse infections have been reported in different areas of Africa, many due to drug resistance (Kupper and Wolters, 1983; Abdel Gadir et al., 1981; Jones-Davies, 1967; MacLennan and Na'Isa, 1970). However, in many areas it is not possible to distinguish between relapse due to resistance or reinfection (Logan et al., 1984). It is possible that relapse infections can occur due to trypanosomes being sequestered in regions that are inaccessible to the drugs, for example, in the aqueous humor and cerebrospinal fluid (Whitelaw et al., 1988) or in the brain (Jennings et al., 1979).

Tsetse flies carrying *T. vivax* can be cured of their infections by ingestion of blood containing isometamidium (Agu, 1984; 1985; Moloo and Kamunya, 1987). This can be effected by allowing infected tsetse to feed for 5 days on animals treated with 1 mg kg^{-1} isometamidium chloride, or on membranes in which blood contains 0.1 mg ml^{-1}. *T. vivax* was eliminated from *G. palpalis* irrespective of whether the infection was mature or developing. Although control of animal trypanosomiasis can effectively be maintained in some areas by drug treatment of livestock, the drawbacks include the expense to the farmer, and the possible induction of drug resistance which appears to be increasing. New drugs for animal trypanosomiasis have not been marketed in the last 30 years.

1.5 Trypanosome organelles

African trypanosomes are "typical" eukaryotic cells possessing a nucleus, ribosomes, endoplasmic reticulum, Golgi apparatus, cytoskeleton, and endocytic organelles. In addition to these, trypanosomes have some morphologically and functionally unique organelles which will be described in this section. An understanding of the differences in trypanosome biology compared to that of the mammalian host is essential before potential differences could be exploited in the design of new chemotherapeutic approaches or vaccines.

1.5.1 The Kinetoplast/Mitochondrion

Trypanosomes have a single mitochondrion that runs the entire length of the cell. The mitochondrion is continuous with a fibrous structure, termed the kinetoplast which houses a complex network of catenated DNA molecules which are grouped as either maxicircles or minicircles (Englund et al., 1982; Stuart, 1983). The maxicircles of *T. brucei* are 20 kb in size, occur as approximately 20-50 identical copies per cell and have coding regions for mitochondrial respiratory proteins (Stuart, 1983; Simpson, 1986). The minicircles are only 1 kb in length but are present in a high copy number of 5,000-10,000 per cell in the catenated network (Simpson, 1986). Minicircles are heterogenous in sequence and appear to be involved in the editing of mitochondrial RNA transcripts by the addition and deletion of uridines (reviewed by Stuart, 1991). The maxicircles of *T. vivax* are similar to those of *T. brucei* in size but have a low degree of homology. *T. vivax* appears to have at least twice as much maxicircle DNA than *T. brucei* (Borst et al., 1985). *T. vivax* minicircle DNA at 465 bp is the smallest minicircle DNA found and has no sequence homology with *T. brucei* minicircles.

The mitochondrion, the site of oxidative phosphorylation in the cell, is elaborated and fully functional in the insect vector stages of *T. brucei*. However, the mitochondrion of bloodstream form *T. brucei* lacks some of the necessary enzymes, therefore glycolysis is the sole energy generating pathway in these cells (Bowman and

Flynn, 1976). The mitochondrion of *T. vivax* bloodstream forms however have tubular cristae and are diaphorase positive which suggests that their mitochondria are more active than that of their *T. brucei* counterparts or perhaps remain activated throughout the life cycle.

1.5.2 Glycosomes

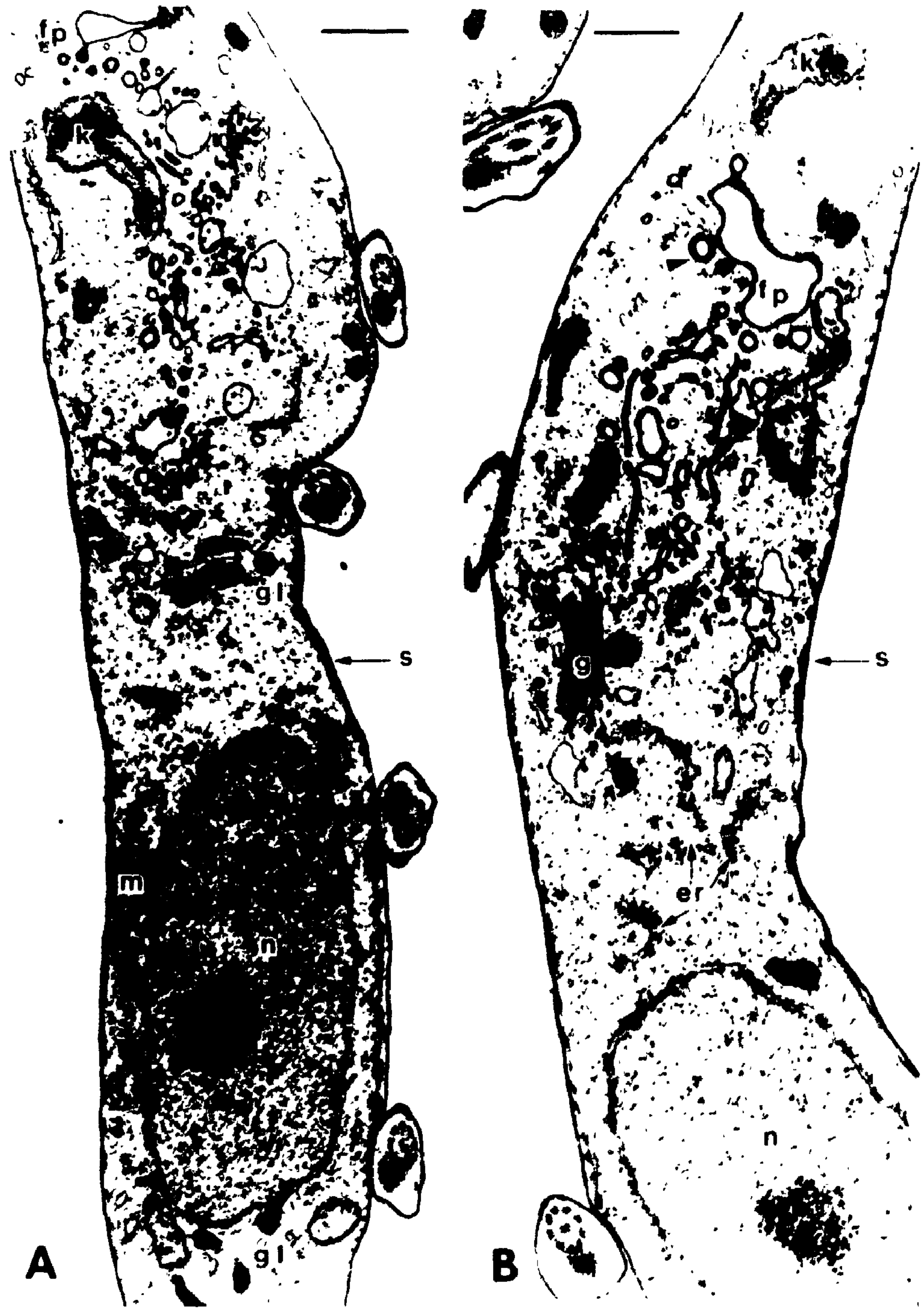
Glycosomes are organelles which are unique to the family Trypanosomatidae (Figs. 1.3). These electron dense organelles (Fig. 1.3 A) contain the first seven enzymes of glycolysis as well as two enzymes involved in glycerol metabolism (Oppordoes and Borst, 1977). Other eukaryotes do not have their glycolytic enzymes compartmentalized as most of these reactions take place in the cytosol. It has been suggested that the trypanosome glycolytic enzymes exist and function uniquely as a multi-enzyme complex within the glycosome (Aman et al., 1985), and perhaps compartmentalization of the enzymes is necessary for this to occur, however, this has not been clearly demonstrated.

Glycosomes and the glycolytic enzymes have been purified and characterized (Opperdoes, 1981; Opperdoes et al., 1984; Misset et al., 1986; Aman and Wang, 1986). *T. brucei* has an estimated 230 glycosomes per cell comprising 4.3% of the total cell volume. The glycolytic enzymes of trypanosomes have a higher isoelectric point in comparison to their mammalian counterparts. Significant biochemical and physical differences were found in trypanosomal hexokinase and phosphofructokinase when compared to the mammalian enzymes. Based on these differences, it has been

Figure 1.3

Ultrastructure of Bloodstream form *T. vivax*

Electron micrographs of epoxy sections through the posterior portion of bloodstream form *T. vivax* ILDat 2.1, harvested from a rat. An electron dense coat covers the entire surface (s) and lines the flagellar pocket (fp). The kinetoplast (k) and nucleus (n) are seen in (A) and (B). The mitochondrion (m) and glycosomes (gl) are labelled in (A) and the Golgi apparatus (g) and rough endoplasmic reticulum (er) are labelled in (B). The arrowhead in (B) indicates a coated vesicle in close proximity to the flagellar pocket. Scale bars = 0.5 µm.


fp
k
gl
s
m
n
gl
A
k
fp
g
s
er
n
B

suggested that it may be possible to design drugs to block the activities of the trypanosomal enzymes (Misset et al., 1986).

1.5.3 Surface Membrane and Flagellar Pocket

Underneath the surface membrane of the trypanosome lie the pellicular microtubules which run roughly longitudinally, maintaining the shape of the cell (Rudzinska and Vickerman, 1968). These mictrotubules, as in higher eukaryotes, are formed by the polymerization of α and ß tubulin monomers which have been extensively characterized in trypanosomes (Gallo and Anderton, 1983; Schneider et al., 1987; Macrae and Gull, 1990; Sherwin et al, 1987; Gull et al., 1986; Gallo and Piregout, 1988). Attached to the cell is the singular flagellum which contains two parallel rod-like structures (Ray et al., 1955). One of these structures is the axoneme which has the typical 9 + 2 arrangement of flagellar microtubules and originates from the basal body (Vickerman, 1969; de Souza and Souto-Padrón, 1980; see Fig. 1.4). The other is the paraflagellar rod, to which no function has yet been attributed but which may simply be a flexible support for the axoneme (Russell et al., 1983; Gallo and Schrével, 1985; Saborio et al., 1989). The only place on the membrane where there are no underlying microtubules, is a specialized area of the surface membrane called the flagellar pocket which is an invagination of the plasma membrane at the site where the flagellum leaves the cell (Vickerman, 1969 and see Fig. 1.4). As early as 1965, it was known that trypanosomes could ingest protein molecules through the flagellar pocket (Brown et al., 1965), but its importance as the only site the parasite has for endocytosis and its role in nutrient uptake has only been a recent appreciation (Langreth and Balber, 1975; Coppens et al., 1987; Webster and Grab, 1988; Webster, 1989; Webster and Fish, 1989). It has also been suggested that the flagellar pocket is the site of exocytosis in the parasite (Barry and Vickerman, 1979) and although quite probable, no experimental evidence has been obtained to support this idea. However, in the related trypanosomatid *Trypanosoma cruzi*, the Ssp-4 molecule, which is expressed on the surface of amastigote forms, appears in the flagellar pocket in differentiating trypomastigotes before it is transported to the surface (Webster et al., 1991).

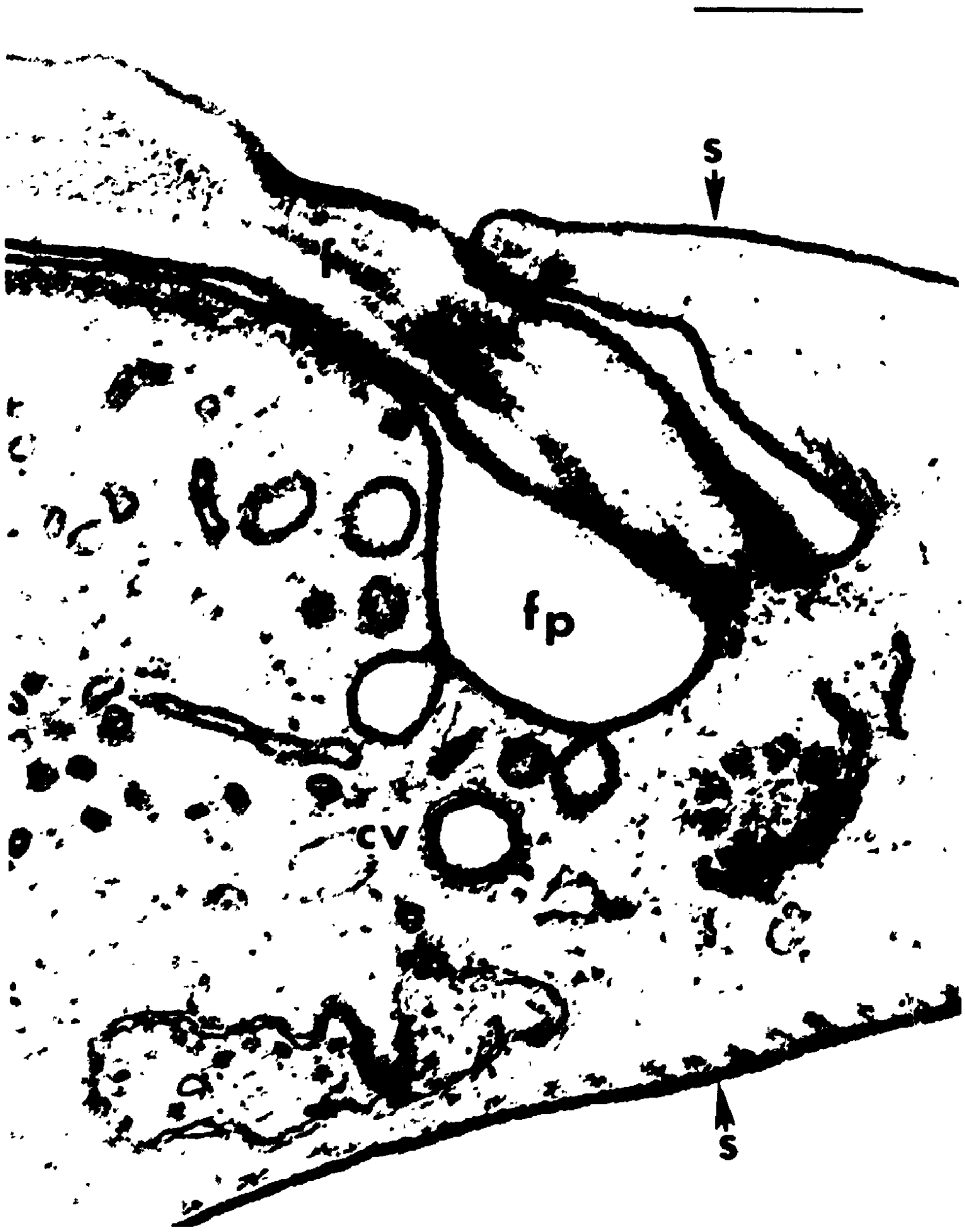
1.5.4 The variant surface glycoprotein

In electron micrographs of sections of bloodstream form trypanosomes, one of the most striking features is the 12-15 nm thick electron dense coat which covers the entire parasite, including the flagellum, and lines the flagellar pocket (Vickerman, 1969; see Figs. 1.3 and 1.4). In *T. brucei*, this coat is made of approximately 1 5 - 2 x 10^7 variant surface glycoprotein (VSG) molecules (Cross, 1975), some of which have been purified and studied extensively. These studies show that *T. brucei* VSGs are glycoprotein molecules of approximately 60 kDa containing 7-17% carbohydrate by weight (Johnson and Cross, 1977) and are anchored to the membrane by a glycosyl-phosphatidylinositol (GPI)-linkage (Ferguson et al., 1988) The membrane form of the VSG, can be converted to a soluble form which lacks the glycolipid anchor (Cardoso de Almeida and Turner, 1983) by the action of an endogenous

Figure 1.4

The flagellar pocket and surface membrane

An electron micrograph of a thin section through the flagellar pocket (fp) region of *T. vivax*. The electron dense surface coat (s), the flagellum (f) and a coated vesicle (cv) can be seen. Scale bar = 0.25 μm.


s
f
fp
cv
s

phosphatidylinositol-specific phospholipase C (PI-PLC) (Ferguson et al., 1985; Jackson arnd Voorheis, 1985; Grab et al., 1987). After the initial sequencing of the N-terminal portion of several VSGs, it was generally accepted that VSGs have highly variable N-terminal sequences (Bridgen et al., 1976; Turner, 1982; Borst and Cross, 1982). In addition, it was assumed that the exposed antigenic sites were located at the N-terminal variable region (Frasch et al., 1980). However, one study involving N-terminal sequence analysis of eight immunologically distinct VSGs isolated from successive peaks of parasitemia of cloned *T. brucei* has shown that a greater degree of homology exists between VSG molecules than originally thought (Olafson et al., 1984). Evidence was also presented to suggest that *T. brucei* VSGs are related to the VSGs of *T. congolense* and *T. equiperdum* through a common primordial gene. A considerable degree of homology exists at the C-terminal end of VSGs and VSGs have been divided into two groups based on these homologies (Rice-Ficht et al., 1981; Holder and Cross, 1981; Matthyssens et al., 1981). In addition, the position of cysteine residues appears to be conserved in families of VSG molecules (Carrington et al., 1991). Most epitopes appear to be buried when the VSG is folded in its native state, probably due to the close molecular packing on the surface and only a few are exposed at the surface as topographically assembled structures (Miller et al., 1984; Clarke et al., 1987; Pinder et al., 1987). Additionally, it was shown that the conserved N-terminal peptides of two serologically cross-reactive VSGs do not contain antigenic determinants (Clarke et al., 1984).

Studies of *T. vivax* VSGs have been hindered by the fact that the majority of isolates only infect ruminants. Few parasites are obtained from infections of cattle and they are rarely homogeneous for VSG expression. Those isolates that will infect rodents are few in number and VSG expression is relatively unstable. However, several VSGs from rodent-adapted clones of *T. vivax* have been identified by surface labelling and their molecular weight range found to be 40-50 kDa, which is smaller than that of the VSGs from *T. brucei* and *T. congolense* (Gardiner et al., 1987). *T. vivax* VSGs have a glycolipid anchor as do the other VSGs and ^{3}H-myristic acid can be incorporated into the VSG (Gardiner et al., 1987) but neither *T. brucei* nor *T. vivax* lysates are able to release the *T. vivax* VSG from membranes (Gardiner et al., 1992), whereas *T. brucei* lysates will release VSG from *T. brucei* membranes. Therefore, the *T. brucei* PI-PLC does not release *T. vivax* VSG and thus the anchor of *T. vivax* VSG may have a different structure than that of *T. brucei*. Recently, a VSG from *T. vivax* ILDat 2.1 was purified and sequence data obtained from both amino acid and DNA sequencing (Gardiner et al., 1992). There is no significant homology between this sequence and that of the VSGs from *T. brucei* and *T. congolense*.

1.6 Antigenic Variation

During the course of a trypanosome infection, the levels of bloodstream parasitemia rise and fall with trypanosome populations expressing antigenically distinct VSGs in a sequential manner. A single trypanosome generally expresses a VSG coat of a single antigenic type, but the trypanosomes comprising each wave of parasitemia are heterogenous with respect to antigen type (McNeillage et al., 1969). Trypanosome populations will express one or a few major antigen types in each wave of parasitemia with minor variants also being expressed. Coincident with the decline of the parasite population, variant antigen type (VAT)-specific antibody is detected in the serum of the host. The next wave of parasitemia will contain variants not yet encountered, or at least responded to, by the host. The rise and fall in parasitemia becomes less pronounced as the infection progresses, but antigenic variation still continues until the death of the animal (Gray and Luckins, 1976). Thus, this mechanism of antigenic variation enables trypanosomes to stay a step ahead of the immune response of the host and contributes to their success as parasites.

Early studies of antigenic variation showed that many antigenic types could be expressed during an infection but that the pattern of arising variants was not random nor was it predictable (Gray 1965; 1975). These studies, using *T. brucei* and a related, human infective subspecies, *T. b. gambiense*, showed that some variants are more likely to appear early in infection than others, even when using different isolates of the same stock and different hosts. Initial studies of antigenic variation involved uncloned trypanosome populations. However, considerable progress in the understanding of the phenomenon, especially the molecular mechanisms, has been made due to the production of clones homogeneous for expression of variant antigen type (VAT) in laboratory rodents. The relationship between the VSG coat and antigenic variation was first suggested by Vickerman and Luckins (1969) and was confirmed after several VSGs were purified and shown to induce variant specific immunity (Cross, 1975; Taylor and Cross, 1977; Fruit et al., 1977).

After cyclical development in the tsetse fly, the metacyclic *T. brucei* populations were observed to revert back to a "basic antigen type" which differs depending on the stock used (Gray, 1965; 1975; Jenni, 1977). Subsequently, it was shown that metacyclic populations are heterogenous with respect to the variant antigen types expressed (Le Ray et al., 1978) but that they are a conserved set of the total repertoire depending on serodeme, and these VATs are independent of the VATs ingested by the tsetse fly (Hajduk and Vickerman, 1981). In addition, it has been shown that the last VAT expressed by the ingested bloodstream form often arises in the first wave of parasitemia following cyclical transmission by the tsetse fly (Hajduk and Vickerman, 1981; Esser et al., 1982; Hajduk, 1984). Thus, although the idea of a single basic type is outdated, trypanosomes do revert to the expression of a limited, repeatable set of variants on transmission by the tsetse fly. The metacyclic VAT (mVAT) repertoire for a particular *T. congolense* serodeme appears to consist of 12-15 different antigen types (Crowe et al., 1983). Multiplying metacyclics can be detected in the extensive chancres characteristic of *T. congolense* infections (Gray and Luckins, 1980) up until day 7 when bloodstream VATs begin to arise (Luckins et al., 1990). In these infections, mVAT-specific antibodies can be detected from 14-35 days following infection. Because the metacyclics are maintained in the skin for so long in *T. congolense* infections, the host is able to develop immunity to the mVATs expressed by the serodeme. Experimental support of this comes from studies where rabbits immunized through infection, then treated 7 days after infection, were immune to homologous challenge (Luckins et al., 1983). Homologous immunity can also be induced in cattle in the same way except that it is necessary to allow the infection to get to the point where bloodstream forms have been established (Akol and Murray, 1985).

The mVATs expressed within each serodeme of *T. brucei rhodesiense* have been followed by sequential transmission of a clone through the tsetse fly vector ten times and the mVATs examined after each transmission (Barry et al., 1983). Most of the mVATs were expressed consistently but one was lost and two new VATs arose at low frequencies. This study suggests that there is a mechanism through which gradual antigenic drift could occur in time. This implies that after several transmissions, the mVAT repertoire may slowly start to change, thus avoiding possible induction of immunity in sequentially challenged animals, as was demonstrated under experimental conditions for *T. congolense*. The "instability" of *T. congolense* mVATs has not been demonstrated to the same degree (Luckins et al., 1990) but it is possible that *T. congolense* may not be as dependent on this type of mechanism for varying the antigenic repertoire.

Antigenic variation has been studied in most detail in *T. brucei* since VAT homogenous populations can easily be grown to large numbers in laboratory rodents, and purification of their VSGs is relatively easy. Comparable studies using *T. vivax* have been hindered since most stocks and clones are restricted to growth in ruminants and, until recently (Gathuo et al., 1987), there have not been antigenically stable, rodent-adapted *T. vivax* clones. In spite of this, several investigators established the existence of antigenic variation in *T. vivax* using rodent-adapted stocks (Clarkson and Awan, 1969; de Gee, 1980; de Gee et al., 1979; 1981) and ruminant infections (Dar, 1972; Jones and Clarkson, 1971). Subsequent studies employed VAT homogenous clones of rodent-adapted *T. vivax* (Barry and Gathuo, 1984; Barry, 1986; Gardiner et al., 1986b). These studies showed that after passage through tsetse flies, the first wave of parasitemia consisted of a mixed population of VATs even when the flies were infected with a cloned population (Gardiner et al., 1986b) In addition, the VAT of the clone which was picked up by the tsetse may not even be expressed in the first peak parasitemia, in contrast to the studies on *T. brucei* in which the last bloodstream VAT expressed before tsetse transmission is often re-expressed in the first bloodstream forms (Hajduk, 1984). Some animals can control and clear their *T. vivax* infections without drug treatment ("self-cure") and most of the animals are subsequently immune to homologous tsetse challenge (Barry, 1986; Nantulya et al., 1986). However, animals that have been treated after a short infection are susceptible to homologous challenge (de Gee, 1980; Emery et al, 1987). Experiments using *T. vivax* metacyclics in attempt to protect animals against homologous challenge have proven disappointing (Vos et al., 1988a), even when large numbers of metacyclics were used for immunization, although this lead to extended prepatent periods (Vos et al., 1988b). Even in animals that had cured their infections and were subsequently immune to challenge, there were no anti-mVAT antibodies produced during infection, so that the immunity to homologous challenge was presumably against bloodstream forms (Nantulya et al., 1986). Obviously, the early presentation of antigenic variants of *T. vivax* to the host is strikingly different to that of *T. brucei* or *T. congolense* and it is possible that mVATs for *T. vivax* do not exist. Alternatively, if mVATs exist which are characteristic for each stock of *T. vivax*, the numbers of metacyclics extruded with a tsetse fly bite may be too low to induce an immune response, or an antigenic switch occurs so rapidly that mVATs are very short-lived in the host. Results from the same studies suggest that the self-cure phenomenon in *T. vivax* results from an exhaustion of the bloodstream form VAT repertoire due to a smaller VSG repertoire and a rapid rate of antigenic variation (Nantulya et al., 1986; Gardiner, 1989). Indeed, the theory of a rapid rate of antigenic variation in *T. vivax* is supported by the unstable expression of VATs, which occurs even in immunosuppressed animals (Barry and Gathuo, 1984).

1.6.1 Genetics of Antigenic Variation

The potential number of antigenically distinct VSGs that could be expressed by a trypanosome is great. *T. brucei* has an estimated 1000 VSG-related sequences (Van der Ploeg et al., 1982) and new VSG sequences can be generated during gene conversion (Pays et al., 1983; Roth et al., 1986) which has been implicated as the major mechanism for antigenic variation in trypanosomes (Aline and Stuart, 1985; Van der Ploeg et al., 1984). VSG genes are transcribed from sites that are immediately upstream of repetitive telomeric sequences and those VSG genes that are resident in intrachromosomal sites must be duplicated and translocated to a telomerelinked site before they are transcribed (Bernards et al., 1981). Since trypanosomes contain a large number of chromosomes, and no telomeres that lack proximal VSG genes have been identified, the potential number of expression sites is large. However, in *T. brucei*, it is now known that only telomeres which contain expression site-associated genes (ESAGs) can be transcribed, thus limiting the number of potential expression sites of bloodstream form VSGs to 14-25 (Cully et al., 1985). The ESAGs belong to a pleomorphic gene family and the function of the products is unknown, but there is evidence that some are membrane glycoproteins (Cully et al., 1986). One ESAG has sequence homology to the yeast adenylate/guanylate cyclase gene (Alexandre et al., 1990), and another encodes a transferrin-binding protein (Schell et al., 1991). An entire expression site from *T. brucei* has been cloned and sequenced (Pays et al., 1989). This expression site contains seven genes in addition to the VSG gene, six of which may encode membrane proteins (Pays et al., 1989). The ESAGs and VSG genes are transcribed as large multi-cistronic units but the mature VSG message, in one specific case, outnumbers an ESAG message 700:1 (Cully et al., 1985). The authors suggest that the difference may arise from alternate transcription initiation at the VSG gene or differential processing of the VSG and ESAG transcripts.

A general model of the mechanism of antigenic variation has been proposed, based on many studies of the molecular basis of VSG expression in *T. brucei* (Pays, 1989). After cyclical transmission in the tsetse fly, the metacyclic VSGs are transiently expressed, preferrably from sites on large chromosomes. This is followed by expression of the first bloodstream form VSG which is generally the one that was expressed last before ingestion by the fly (Hajduk, 1984; Delauw et al., 1985). This probably results from the continuous activity of the VSG gene promoter which lies very far upstream and appears to be active throughout the parasite life cycle (Pays et al., 1989). Early antigen types will then be expressed. The genes for these VSGs would be telomeric and expression of them would involve *in situ* activation and no DNA rearrangement (Young et al., 1982; Laurent et al., 1984). The next in the sequence of VSG genes to be expressed would be the non-telomeric genes. This requires duplication of a VSG gene and its non-reciprocal recombination into the expression site. Frequent recombination of genes with expression sites seems to depend on the number of homologous sequences which occur outside the coding sequence. The flanking sequences are 70 base pair repeats of variable number (Aline et al., 1985), making some VSG genes less likely to be expressed than others. If no regions homologous to the expression site are available in the unexpressed gene, but internal homologies exist with a gene that is currently in an expression site, internal recombination could occur, leading to chimeric genes which will contribute to the antigenic repertoire of the trypanosome. In addition, using a similar mechanism, it is possible that pseudogene-like regions may be used in recombination to form new VSG genes (Roth et al., 1986; Thon et al., 1989) This would occur with low probability and such events seem to occur quite late in infection (Pays et al., 1985; Longacre and Eisen, 1986). This molecular model fits the early serological studies where it has been observed that some VATs will arise early and some later and some are more likely to be expressed than others during infection (Gray, 1965).

1.6.2 Implications of Antigenic Variation

Due to the sequential expression of a potentially large number of antigenically distinct VSG molecules during the course of a trypanosome infection, it is generally accepted that a vaccine based on VSG would be unsuitable for protective immunization against heterologous VATs. Although there are some conserved areas between different VSG molecules of the same species, these cannot be exploited since they are not exposed in the native state on the surface of the cell (Miller et al., 1984, Clarke et al., 1987; Pinder et al., 1987). The metacyclic VSG repertoire is much smaller than the bloodstream form repertoire, 12-15 mVATs within a serodeme. However, a vaccine based on mVATs is also impractical since the number of serodemes of each species, although not defined, is large and antigenic drift can occur Further, this approach would be ineffective for immunization against *T. vivax* since no protection is afforded when metacyclics are used for immunization (Vos et al., 1988 a, b). For the reasons outlined, a VSG-based vaccine will not work and it has become necessary to approach the problem of identifying potential vaccine candidates from a different perspective. To this end, the recent focus in trypanosome research has been directed toward the identification of invariant molecules that may be vulnerable to immune attack.

The bloodstream forms of trypanosomes are obligate parasites and are dependent on the uptake of nutrients from the host for their survival. For example, these parasites have an absolute requirement for glucose, which is taken into the cell by a carrier-mediated system (Eisenthal et al., 1989), and for iron (ILRAD, unpublished observations) which is probably brought into the cell via transferrin as it is in mammalian cells (Klausner et al., 1983a, b). In order for the trypanosome to interact with the extracellular milieu, the blood, it must have cell surface receptors and transporters for the specific uptake of nutrients. The tightly packed VSG surface coat covers the entire parasite and lines the flagellar pocket membrane. The barrier that VSG presents would appear to be impenetrable since buried epitopes on VSG molecules are inaccessible to antibody binding. However, if protein ligands such as transferrin and low density lipoprotein (LDL) are able to bind to specific receptors (Coppens et al., 1987), it may be possible that antibodies directed to these or similar receptors may be useful for blocking the uptake of nutrients which may in turn have a detrimental effect on the parasite. Alternatively, bound antibody may be able to fix complement and the trypanosomes would be lysed. Therefore, the initial step in this type of strategy is to identify possible targets for immune attack. The favoured candidates would be invariant membrane proteins on the cell surface, flagellar pocket, or in components of the endocytic pathway.

1.7 Invariant Trypanosomal Proteins

Several invariant, surface, or flagellar pocket proteins have been identified in trypanosomes, either by direct methods including purification and antibody production, or indirectly, where their presence has been inferred due to a measurable activity or a DNA sequence. Some of these invariant proteins that may have potential as targets for immune intervention will be described below.

An enzyme which has two catalytic activities: a 3'-nucleotidase activity and a calcium-dependent endoribonuclease activity, has been shown to be distributed over the cell surface and the flagellar pocket of trypanosomes (Gardiner et al., 1982; Gbenle et al., 1986; Gottlieb et al., 1986). A similar enzyme from *Crithidia luciliae* has been purified and well characterized and its expression was shown to be inducible upon purine starvation (Gottlieb et al., 1988; Neubert and Gottlieb, 1990). Other possible target proteins of *T. brucei* include an epidermal growth factor receptor homologue (Hide et al., 1989), a glucose transporter homologue (Bringaud and Baltz, 1992), a low-density lipoprotein receptor (Coppens et al., 1991), a cysteine-rich acidic integral membrane protein which has been localized to the flagellar pocket (Lee et al , 1990), a transferrin-binding protein (Schell et al., 1991), a transmembrane protein associated with the flagellum attachment region (Woods et al., 1989), various flagellar pocket antigens (McLaughlin, 1987) and two invariant surface antigens, ISG65 and ISG75 recently characterized by Ziegelbauer et al. (1992). Very little is presently known about most of these proteins in terms of degree of exposure to the environment, accessibility to antibody, or usefulness as a potential vaccine. However, immunization with a purified flagellar pocket membrane preparation provided some protection against homologous infection with the same clone and the stock from which the clone was derived (McLaughlin, 1987), as well as clones of distinct variant antigen types (Olenick et al., 1988). An 83 kDa surface membrane protein was identified in *T. brucei* and *T. vivax* (Rovis et al., 1984), but after immunization of mice with this protein and induction of high antibody titres, no protection was afforded against tsetse-transmitted challenge with either parasite. Therefore, the number of invariant surface and/or flagellar pocket antigens which have been described for trypanosomes is limited and even less data exist as to their usefulness as vaccine candidates.

Another logical site for potential immune intervention is the endocytic pathway. Proteins which are resident in endocytic organelles presumably come in contact with endocytosed material. Since trypanosomes ingest serum proteins including immunoglobulins (personal observations), it may be possible to immunize animals with purified proteins that are part of the endocytic pathway, with the hope that ingested trypanosome-specific antibody may be able to bind to the target molecule and interfere with its function. Proteins which are located in an endocytic compartment that have been described to date, are a 77 kDa membrane protein associated with coated vesicles and early endosomes in *T. brucei* (Webster and Shapiro, 1990), and a *T. congolense*-specific lysosomal cysteine protease (Mbawa et al., 1991). Immunization of mice with the 77 kDa protein did not protect these animals against infection with *T. brucei* (P. Webster, personal communication). The cysteine protease is an interesting molecule since it can be used for a marker of lysosomes in *T. congolense* and may have potential use as a target for immune intervention. In addition, experiments which examined the immune responses of both trypanotolerant and susceptible cattle to *T. congolense* infections demonstrated that during the course of infection, trypanotolerant cattle consistently produced antibody responses to the cysteine protease but that susceptible cattle did not (E. Authie, personal communication). Therefore, the cysteine protease may be significant in the elucidation of an immune response that confers tolerance to trypanosome infection

CHAPTER 2

OBJECTIVES AND RATIONALE

Trypanosoma vivax was the focus of this study for two reasons. First, as outlined in Chapter 1, *T. vivax* is an important animal pathogen in both Africa and South America. As opposed to *T. brucei*, which is widespread in the field but causes rare pathology in livestock (Killick-Kendrich, 1971), no biochemical studies have been carried out using *T. vivax*, apart from the recent characterization of several VSGs (Gardiner et al., 1987; Gardiner et al., 1992). Investigators tend to choose *T. brucei* as a model system to carry out their studies since it is easier to manipulate in the laboratory. *T. vivax* is difficult to handle due to the comparatively low numbers obtained in rodent infections and its fragility when isolated from blood. In addition to having more relevance in the field, *T. vivax* bloodstream forms have a surface coat which may be more loosely arranged than that of its counterparts, *T. brucei* and *T. congolense*, and thus the underlying surface molecules may be more accessible to immune attack. Evidence to suggest that the coat of *T. vivax* bloodstream forms may be more loosely arranged includes the observation that it can appear less dense in thin sections examined by electron microscopy and that *T. vivax* is susceptible to lysis by aerolysin, whereas the other species are not (Gardiner et al., 1987).

The rationale behind developing a vaccine for animal trypanosomiasis lies in the exploitation of invariant proteins that would be accessible to antibody binding on live trypanosomes. Of the invariant membrane proteins that have been identified in

T. brucei, few have been characterized in terms of accessibility to antibody binding. If the surface coat of *T. vivax* is more loosely arranged, this should increase the chances that underlying proteins are exposed or accessible to antibody binding. This concept can be extended to include invariant membrane components of the endocytic pathway which may similarly come into contact with exogenous reagents including antibodies. Therefore, well characterized rodent-infective *T. vivax* stocks and clones were used to try to identify invariant membrane proteins of this species. As an approach to this study, the following objectives were set:

1. To raise antibodies specific for invariant membrane proteins which are localized to the cell surface, flagellar pocket or endocytic organelles.
2. Immunoaffinity purification, biochemical characterization, and N-terminal sequencing of the protein.
3. Detailed immunolocalization of the protein using the specific antibody(ies)
4. Determination of the accessibility of the protein to antibody binding and the effect of antibody binding on viability.

The first objective of the study was to raise antibodies which could aid in the identification of potential invariant membrane proteins, one of which would be further characterized if suitable *ie*: cell surface, flagellar po[illegible] endocytic pathway. The initial approach to this objective was to raise polyclonal antisera to an enriched plasma membrane fraction (following the protocol of Rovis and Baekkeskov, 1980), from bloodstream forms of *T. vivax*. Polyclonal antisera raised in rabbits contained antibodies that reacted with several invariant proteins, after the first boost, but the predominant antibody response was directed to VSG. Subsequent boosts using similarly prepared membranes succeeded only in producing high titre anti-VSG antisera.

Since a better purification procedure for surface membranes was not found, the production of monoclonal antibodies to *T. vivax* was used to obtain specificity. To avoid the problem of an overwhelming anti-VSG response, three day, culture forms of *T. vivax*, lacking an intact VSG coat, were chosen for immunization of Balb/c mice. Since relatively small numbers of uncoated forms could be obtained, membranes were not prepared and immunization was carried out using whole, formalin fixed cells. It was also hoped that this method of antigen presentation would increase the probability of producing an antibody response to exposed surface molecules.

Eleven monoclonal antibodies (mAbs) were produced with various specificities. Many of these mAbs were specific for VSG, possibly due to residual VSG non-specifically associated with the surface of differentiated cells, as the culture form parasites were not washed extensively before fixation and use in immunization due to their fragility. After discarding the mAbs specific for VSG and those that recognized more than one protein, two monoclonals were selected for use in further investigations: mAb 4E1, which was found to be specific for a 65 kDa vesicle-associated protein; and mAb 4B11, which was specific for an invariant surface membrane protein of 35 kDa. Although studies were initiated using mAb 4B11, and these are discussed briefly in Chapter 6 in relation to the *T. vivax* 3'-nucleotidase, this mAb, due to its instability, could not be used to its full potential in the characterization of the 35 kDa antigen. Consequently, studies were then initiated using mAb 4E1 which was specific for an invariant 65 kDa antigen. The characterization of gp65 and the description of its localization within an intermediate compartment of the endocytic pathway in *T. vivax* is the subject of Chapters 3 to 5.

CHAPTER 3

Identification and Partial Characterization of a 65 kDa Antigen (gp65) in *Trypanosoma vivax*.

3.1 Introduction

In order to identify an invariant membrane protein of *Trypanosoma vivax*, as an initial step toward the eventual assessment of this protein as a potential target for immune intervention, monoclonal antibodies were raised to whole fixed uncoated culture forms of *T. vivax*. One of the monoclonal antibodies obtained, mAb 4E1, was used to identify and partially characterize an internal membrane glycoprotein (gp65) which was localized to the posterior region of the trypanosome. In this chapter, the raising of monoclonal antibodies to invariant proteins of *T. vivax* and the partial biochemical characterization of gp65 is described.

3.2 Materials and Methods

3.2.1 Trypanosomes

Bloodstream forms. *Trypanosoma (Duttonella) vivax* IL 1392, a naturally rodent-infective stock, was derived from Y486, a stock originally isolated from a steer in Zaria, Nigeria (Leeflang et al., 1976). All clones used were homogenous for expressed variant antigen type (VAT). The derivation of ILDat 1.2, a clone of IL 1392, has been described (Barry and Gathuo, 1984). *T. vivax* ILDat 2.1 is a rodent-adapted clone of a stock isolated in Lugala, Uganda (Gathuo et al., 1987). *T. vivax* IL 3067, is a stock isolated from Bamburi, Kenya in 1986 and *T. vivax* Palmira is a primary isolate from the Cauca Valley, Colombia, South America (Dirie et al., 1992). As these latter two isolates were restricted to growth in ruminants, they were raised in Boran cattle following intravenous (i.v.) inoculation of a cryopreserved stabilate. *T. brucei brucei* GuTat 3.1 was derived from Treu 667 stock and cloned in Balb/c mice and its properties have been described by Sendashonga and Black (1982). *T. congolense* IL 3000 (Fish et al., 1989), was cloned from stock IL 2985 a derivative of stock C-49 (Wellde et al., 1974). Bloodstream forms of *T. vivax*, except IL 3067 and the Palmira isolate, were grown in sublethally irradiated (600 rad.) adult Swiss mice or Sprague Dawley rats, after an intraperitoneal (i.p.) inoculation of cryopreserved stabilates. *T. brucei* and *T. congolense* bloodstream forms were raised in irradiated rats. Parasitemias were detected by wet blood film examination or by the haematocrit centrifugation technique (Woo, 1970) for infections of cattle. Whole blood was collected just before the peak of parasitemia (~5 x 10^8 parasites/ml) and diluted with an equal volume of phosphate-buffered saline glucose (PSG; pH 8.0) [38 mM Na_2HPO_4, 2 mM NaH_2PO_4-$2H_2O$, 29 mM NaCl, 1% (w/v) glucose for mouse and bovine blood; 57 mM Na_2HPO_4, 3 mM NaH_2PO_4-$2H_2O$, 43.5 mM NaCl, 1% (w/v) glucose for rat blood] supplemented with 0.1 mM hypoxanthine (Lonsdale-Eccles and Grab, 1987a). For separation of trypanosomes from blood cells, diluted blood was passed over a DE-52 column (Lanham and Godfrey, 1970) equilibrated with PSG/hypoxanthine for equilibration and elution.

Culture forms. The generation of 3 day uncoated culture forms, epimastigotes and metacyclics of *T. vivax* IL 1392 was accomplished as described by Gumm (1991). Uncoated culture forms were produced *in vitro* as follows. Bloodstream forms of *T. vivax* IL 1392 were harvested from mice at high parasitemia (10^9 cells/ml). Cultures were seeded and maintained at 27°C in the medium described (Gumm, 1991). On the third day, during the transformation of bloodstream forms to epimastigote forms, there exists a non-dividing, non-infective form of the parasite that lacks an intact VSG surface coat. These are referred to as three day, uncoated, culture forms. Procylic forms of *T. brucei* GuTat 3.1 were established from the cloned bloodstream form (Brun and Shönenberger, 1979). These and *T. congolense* culture forms were maintained in culture at 27°C in Eagles MEM containing 25 mM Hepes, Earle's salts and 2 mM glutamine with the following additions: 10% (v/v) fetal bovine serum (FBS), 1% (v/v) non-essential amino acids, 60 mM proline, 6.5 µg/ml hemin and 200 µM hypoxanthine.

3.2.2 *Leishmania donovani*.

Leishmania donovani NLB-065 is a recent human isolate obtained as promastigotes from Dr. J. Githure (KEMRI) via Dr. T. W. Pearson (University of Victoria, B.C.). It was maintained in culture in the same medium and under the same conditions as the *T. brucei* procyclics.

3.2.3 Monoclonal Antibodies.

Three day, uncoated, culture forms of *T. vivax* IL 1392 were fixed for 2 hours in 4% (w/v) paraformaldehyde in 100 mM Pipes (pH 7.0) at room temperature. Fixed cells were washed in Dulbecco's PBS (DPBS; pH 7.4) [8.1 mM Na_2HPO_4-$7H_2O$, 1.2 mM KH_2PO_4, 138 mM NaCl, 2.7 mM KCl, 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$-$6H_2O$] three times by centrifugation (750*g*, 10 minutes) then remaining free aldehyde groups were quenched for 30 minutes using 50 mM NH_4Cl in DPBS. Balb/c mice were injected with 2 x 10^7 fixed parasites emulsified with complete Freund's adjuvant (CFA) for primary immunization and incomplete Freund's adjuvant (ICFA) for subsequent boosts. Three injections were given at 21 day intervals. Test serum was obtained after collection of blood from the tail vein and used in immunofluorescence on thick cryosections of *T. vivax* bloodstream forms (see below) and western blotting When invariant antigens were detected by western blotting and surface labelling was apparent by immunofluorescence, the tested mouse was given a further i.v. injection of 2 x 10^7 fixed trypanosomes in DPBS, four days prior to removal of the spleen for fusion. Fusion of mouse spleen cells with P3-X63-Ag8 mouse myeloma cells (Köhler and Milstein, 1975) was carried out essentially following the methods of Pearson et al (1980). Minor adjustments were made in the protocol in that hypoxanthine/ aminopterin/thymidine (HAT) was added to the fusion medium together with mouse thymocytes at a concentration of 2.5 x 10^6 cells/ml immediately following the fusion From 10 days after fusion, tissue culture supernatants, from wells positive for hybridoma growth, were screened in the first instance by immunofluorescence on fixed, cryosections of *T. vivax* IL 1392 bloodstream forms and secondly by immunoblotting. Eleven hybridomas that were secreting antibodies with different reactivities were doubly cloned by a limiting dilution method. After cloning, on the basis of immunofluorescence screening and immunoblot analysis, mAb 4E1 and mAb 4B11 (discussed in Chapter 6) were selected. The isotype of the monoclonal antibodies was determined using a mouse monoclonal isotyping kit (Amersham). mAb 4E1 was found to be an IgG_1 and mAb 4B11 was an IgM, and both had kappa light chains.

3.2.4 Polyclonal Antisera.

Anti-ILDat 2.1 VSG (RαVSG) antisera was raised in rabbits (bred on the ILRAD farm) against the variant surface glycoprotein (VSG) of *T. vivax* ILDat 2.1. VSG was purified by excision and electroelution from preparative SDS-PAGE gels of whole trypanosome lysates. Electroelution of protein from minced gel slices was carried out in an electroeluter/concentrator (Model #ECU-040, C.B.S. Scientific Co., Del Mar, California) for 18 hours at 12 mA per cell in 50 mM NH_4HCO_3 (pH 8.2) containing 0.01% (w/v) SDS. Recovered protein was dialyzed against 4 litres of DPBS overnight at 4°C and then lyophilized. 100 μg of VSG was emulsified with complete Freund's adjuvant (CFA) and injected subcutaneously at multiple sites along the backs of 2 rabbits. Three injections of 50 μg of VSG, emulsified in incomplete Freund's adjuvant (ICFA), were used to boost the rabbits. Rabbits were bled by cardiac puncture and antisera was obtained from clotted blood.

3.2.5 Methods for Screening Tissue Culture Supernatants

A) Immunofluorescence using thick cryosections of *T. vivax* ILDat 1.2 bloodstream forms. Bloodstream form *T. vivax* ILDat 1.2, a clone derived from the stock IL 1392 which was used to immunize mice for the raising of the monoclonal antibodies, were fixed with 4% (w/v) paraformaldehyde for 1 hour and then pelleted at 10,000*g* for 5 minutes. The pellet was cut into small pieces and infiltrated with 2.3 M sucrose in DPBS for at least 60 minutes at room temperature. Small pyramid-shaped pieces were mounted on copper studs, frozen and stored in liquid nitrogen. Thick cryosections (300 nm) were cut at -60°C using tungsten coated glass knives (Roberts, 1975) and an MT-2B ultramicrotome fitted with a FTS-cryoattachment (Sorvall/RMC Inc., USA), following the techniques described for ultra-cryomicrotomy (Griffiths et al., 1983; Griffiths et al., 1984). Sections were placed on slides coated with 0.1 mg/ml poly-L-lysine. For immunofluorescence, the quenching, washing and diluting buffer was DPBS containing 5% (v/v) FBS and 50 mM NH_4Cl. Slides, to which sections had adhered, were incubated in wash buffer for 30 minutes then incubated in neat tissue culture supernatant for 30 minutes in a humidified chamber. Slides were then washed, 3 times for 5 minutes each wash, and incubated in FITC-conjugated anti-mouse Ig (Amersham) at a 1:50 dilution in wash buffer for 30 minutes in a humidified container, and finally washed for 3 times 5 minutes. The sections were never allowed to dry throughout the procedure or before examination under the light microscope. A drop of Citifluor™ (Agar Scientific, Stanstead, U.K.), an anti-fade compound, was placed onto the section and overlaid with a cover slip and examined using an Axiophot fluorescence microscope (Carl Zeiss, Germany).

B) Immunofluorescence on fixed permeabilized *T. vivax*. *T. vivax* trypanosomes, ILDat 2.1 harvested from rats and purified by DE-52 chromatography, were suspended in DPBS at a concentration of 4 x 10^7 cells/ml. They were fixed by adding an equal volume of 4% (w/v) paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.0) for 1 hour at room temperature. Cells were washed twice by centrifugation at 750*g* for 10 minutes and resuspended in DPBS. Fixed cells were diluted in DPBS and cytospin preparations of 10^5 cells were made by spinning the cell suspension for 10 minutes at 2,000 r.p.m. onto slides coated with 2% (v/v) 3-aminopropyltriethyl-silane in acetone, using a cytospin apparatus (Shandon Southern Products Ltd.). Slides were incubated for 1 hour in 1% (v/v) glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.0) and washed for 10 minutes in DPBS containing 0.1% (w/v) BSA and 50 mM NH_4Cl (wash buffer). Some of the cells were permeabilized using 0.1% (v/v) Triton X-100 in DPBS for 2 hours at room temperature. Remaining free aldehyde groups were quenched by washing twice in wash buffer for a total of 30 minutes. 50 µl of mAb 4E1 (diluted to 1:5000 in wash buffer) was spread over the cells and incubated 1 hour at room temperature. Cells were washed for 30 minutes then incubated for 1 hour at room temperature in FITC-conjugated sheep anti-mouse immunoglobulin (Ig) at a final dilution of 1:50 and then washed twice in wash buffer and once in DPBS. Permanent mounts were made with

Citifluor™ anti-fade mounting compound and cells were examined with the fluorescence microscope and photographs taken with 100 Tmax film (Kodak).

C) Immunoblotting. After cells were harvested from either infected blood or culture, they were pelleted by centrifugation at 750*g* and lysed in boiling Laemmli sample buffer (Laemmli, 1970) at a concentration of approximately 10^8 cells/ml without reducing agents or bromphenol blue. After boiling for 5 minutes, insoluble aggregates were removed by centrifugation at 20,000*g* for 15 minutes. Protein concentration was determined by the method of Lowry et al. (1951) using BSA as a standard. SDS-PAGE separation of trypanosome proteins was carried out as described by Laemmli (1970), on 1.0 mm, 10-15% gradient slab gels with a 4% stacking gel unless otherwise stated. Proteins were partially renatured by incubating the gel in 50 mM Tris (pH 7.4), 20% (v/v) glycerol, for 1 hour prior to electrophoretic transfer onto Immobilon-P™ (Millipore) at 20V at 4°C for 18 hours (Dunn, 1986). Electroblotting buffer consisted of 10 mM NaH_2CO_3, 3 mM Na_2CO_3 (pH 10), 20% (v/v) methanol (Dunn, 1986). The blots were rinsed once with 10 mM Tris (pH 7.4), 150 mM NaCl, 0.1% (v/v) Tween-20 (TBST). Non-specific protein binding was blocked for 1 hour at room temperature using 5% (w/v) low fat skim milk buffered with 10 mM Tris (pH 7.4), 15 mM NaCl. After blocking, the blots were rinsed twice with TBST, then incubated in mAb 4E1 IgG, diluted 1:5000 in 5% (v/v) skim milk solution, for 1 hour at room temperature. Blots were washed with 5 changes of TBST over 30 minutes. The blots were incubated in 2 µCi ^{125}I anti-mouse Ig (Amersham), unless otherwise stated, in 20 ml skim milk solution for 1 hour, followed by extensive washing in TBST. Blots were dried and exposed to Fuji RX X-ray film for autoradiography at -70°C.

3.2.6 Preparation of Colloidal Gold Probes.

Monodisperse gold sols of homogeneous sizes (5 nm, 10 nm, and 15 nm) were produced according to the method of Slot and Geuze (1985). Both 10 nm and 15 nm gold sols were coupled to staphylococcal protein A (Sigma) (protein A-gold--PAG) and 5 nm and 10 nm gold sols were coupled to bovine serum albumin (BSA-gold) using the method of Horisberger et al., (1985).

3.2.7 Immunolabelling of thin cryosections of *T. vivax* ILDat 2.1.

Essentially the same protocol was used for the preparation of *T. vivax* ILDat 2.1 bloodstream forms as was described in Section 2.2.5 A, with the following exceptions. Trypanosome suspensions were fixed by adding an equal volume of 1% (v/v) glutaraldehyde in 200 mM Pipes (pH 7.0). Ultrathin sections (~70 nm) were cut at -110°C and the frozen sections were removed from the surface of the knife with a drop of 2.3 M sucrose in DPBS (pH 7.4), thawed and placed onto formvar/carbon coated copper or nickel grids.

Thawed sections were labelled by incubation with mAb 4E1 tissue culture supernatant (at a dilution of 1:100 for mAb 4E1) for 1 hour at room temperature in a humidified chamber, by floating the grids on 5 µl drops of diluted tissue culture supernatant. The sections were washed for 15 minutes with DPBS/FBS, then incubated with a polyclonal rabbit anti-mouse Ig antiserum (a gift from P. Webster, ILRAD) for 30 minutes at room temperature. Sections were washed as described above and then incubated with 10 nm PAG for 15 minutes at room temperature and washed for 30 minutes in DPBS/FBS and then for 5 minutes in distilled H_2O. Sections were stained for 10 minutes using a 9:1 ratio of 2% (w/v) methyl cellulose and 3% (w/v) uranyl acetate. Excess methyl cellulose/uranyl acetate was drained off by touching the side of the grid to filter paper. After drying, the grids were examined using an EM10A electron microscope operated at 80 kV (Carl Zeiss, Oberkochen, Germany).

3.2.8 Preparation of ascites fluid and purification of mAb 4E1 IgG.

Adult Balb/c x Swiss mice were given an i.p. injection of 50 μg pristane in DPBS ten days prior to an i.p. injection of 5×10^5-10^6 hybridoma cells suspended in 500 μl DPBS. Ascites fluid was collected between 7 and 14 days after injection, pooled and centrifuged at 750*g* for 15 minutes to remove cells and the supernatant was stored at -20°C. Ascites fluid obtained from mice was tested separately for antibody and titrated by immunoblotting using *T. vivax* antigens.

For purification of IgG from pooled ascites fluid, the following standard procedure was followed (Harlow and Lane, 1988). Ascites fluid was brought to 50% saturation of $(NH_4)_2SO_4$ by the dropwise addition of a saturated solution of $(NH_4)_2SO_4$ (pH 7.0) and kept at 4°C overnight. Precipitated proteins were pelleted by centrifugation at 3000*g* for 30 minutes and the supernatant discarded. The pellet was dissolved in 20 mM Tris-HCl, (pH 8.5), 20 mM NaCl and dialyzed at 4°C against three changes of 4 litres of the same buffer. DE-52 cellulose was suspended in the same buffer, brought to pH 8.5 by the addition of concentrated HCl, filtered and washed. A 1.2 cm x 8 cm column of DE-52 was poured and the matrix was equilibrated overnight with 50 column volumes of buffer. The dialyzed antibody solution was loaded onto the column, unbound proteins were washed off until the absorbance at 280 nm was negligible, then a gradient of 20 mM-500 mM NaCl in 20 mM Tris (pH 8.5) was used to elute the bound proteins. Aliquots of fractions collected were run on an SDS-PAGE gel. Those fractions containing the most IgG were pooled and the activity of mAb 4E1 was titrated on immunoblots. All of the experiments described in this and subsequent chapters involving mAb 4E1 were carried out using the IgG fraction purified from ascites unless otherwise stated.

3.2.9 Subcellular Fractionation.

Bloodstream forms of *T. vivax* ILDat 2.1 were washed once in DPBS containing 0.1% (w/v) glucose then suspended in ice-cold SHK buffer: 50 mM Hepes (pH 7.4), 250 mM sucrose, 25 mM KCl (Grab et al., 1987), with 100 µg/ml of each of the protease inhibitors, E-64, leupeptin, and antipain (Cambridge Biochemicals) (Londsdale-Eccles and Mpimbaza, 1986). The cells were disrupted in an ice-cold French pressure cell at 2500 p.s.i., collected on ice and EDTA was added to a final concentration of 1 mM. Unbroken cells and nuclei were removed by centrifugation at 750g for 15 minutes. The post-nuclear supernatant was subjected to differential centrifugation as follows: centrifugation at 10,000*g* for 15 minutes, and the resultant supernatant was spun at 35,000*g*, for 15 minutes. Both spins were carried out using a JA-17 rotor in a Beckman Model J2-21M centrifuge. The 40,000*g* supernatant was then centrifuged at 120,000*g* for 1 hour using a Beckman 42.1Ti rotor in a Beckman Model L8-M ultracentrifuge, resulting in a pellet and a high speed supernatant (HSS) fraction. The protein content of the HSS and the pellets from each centrifugation step were determined using the Lowry assay. To further separate membranes on the basis of density, the 120,000*g* pellet was gently resuspended in SHK buffer containing 1 mM EDTA (SHKE) and 100 μg/ml of each protease inhibitor, then diluted to a concentration of 4 mg/ml protein. One ml of the membrane suspension was gently overlaid onto an 11 ml linear sucrose gradient of 15-40% sucrose in SHKE buffer in a cellulose nitrate tube and centrifuged at 100,000*g* in an SW 41 rotor for 15 hours at 4°C. Fractions (500 μl) were collected from the lowest to the highest sucrose concentration using a Buchler Auto Densi-Flow IIC fraction collector. The refractive index and sucrose concentration of each fraction was determined using a refractometer and the protein content was measured in a spectrophotometer at 280 nm. Protein from 50 μl aliquots of selected fractions was precipitated using the chloroform/methanol/ water method (Wessel and Flügge, 1984). Precipitates were dissolved in Laemmli sample buffer by boiling for 5 minutes and analyzed by SDG-PAGE and immunoblotting.

3.2.10 Preparation of Crude Membranes.

Essentially the same procedure was used as described in the subcellular fractionation method except that the post-nuclear supernatant was centrifuged at 120,000*g* using a Beckman 42.1 Ti rotor for 1 hour. The resultant pellet fraction is referred to as the crude membrane fraction.

3.2.11 Sodium Carbonate Treatment of Crude Membranes.

Freshly prepared crude membranes were suspended in 10 mM Tris, (pH 8.0), 150 mM NaCl (TBS), then diluted 20-fold using ice-cold 0.2 M Na_2CO_3, essentially following the protocol of Fujiki et al., (1982). After incubating the membranes on ice for 1 hour, the samples were centrifuged for 1 hour at 120,000*g* and both the supernatant and pellet fractions kept. The pellets were rinsed with ice-cold distilled H_2O then suspended in TBS (pH 7.4) with 50 µg/ml each of the protease inhibitors. The supernatant fraction was dialyzed overnight against 4 litres of TBS (pH 7.4) at 4°C and then concentrated 10-fold using a Centriprep-10 concentrator (Millipore). Protein concentrations were determined using the Lowry assay (Lowry, 1951).

3.2.12 Triton X-114 Phase Separation of Membrane Proteins.

Crude membranes were solubilized in 2% (v/v) pre-condensed Triton X-114 (Sigma) in TBS (pH 7.4), on ice for 1 hour with occasional mixing. Insoluble material was removed by centrifugation at 120,000*g* at 4°C for 1 hour. Phase separation was performed at 37°C according to Bordier (1981). The detergent phase was separated from the aqueous phase by centrifugation at 3,000*g* for 5 minutes at 30°C. Aliquots of both the detergent and aqueous phases were used for protein determination and analysis by SDS-PAGE and immunoblotting with mAb 4E1

3.2.13 Lectin Precipitation.

T. vivax ILDat 2.1 bloodstream forms were washed once in DPBS with 0.1% (w/v) glucose and lysed at a concentration of 5 x 10^8 cells/ml in 10 mM Tris (pH 7.4), 150 mM NaCl, 1 mM $MnCl_2$, 5 mM $CaCl_2$, 2% (v/v) NP-40 with 100 μg/ml of each of the protease inhibitors antipain, leupeptin and E-64. The cells were frozen in liquid nitrogen and thawed at 37°C (two cycles) and insoluble material was removed by centrifugation at 100,000*g* in a 42.1 Ti rotor (Beckman Instruments) for 1 hour at 4°C. The detergent soluble supernatant was mixed for 1 hour at room temperature with one-tenth volume of packed Concanavalin A (Con A)-Sepharose (Pharmacia) beads or *Ricinus communis* agglutinin I (RCA)-agarose (Vector Laboratories) which had been pre-equilibrated with the lysis buffer. Unbound material was removed by centrifugation at 750*g* for 5 minutes (Beckman TJ-6 centrifuge) and the beads were washed at least 5 times with 10 volumes of lysis buffer before eluting bound glycoproteins with 0.5 M α-methyl mannoside for Con A or 0.5 M galactose for RCA, both in 10 mM Tris (pH 7.4) containing 150 mM NaCl, 0.1% (v/v) NP-40, and 50 μg/ml protease inhibitors. The elution buffer was added to the beads in a 1:1 (v/v) ratio and incubated at 37°C for 15 minutes with occasional mixing. Eluted proteins were dialyzed at 4°C overnight against 4 litres TBS (pH 7.4) then concentrated using a Centriprep-10 concentrator and stored at -80°C.

3.2.14 Glycosidase Digestions.

Proteins eluted from RCA-agarose, 15 µg for each treatment, were precipitated y the chloroform/methanol/water method (Wessel and Flügge, 1984). Protein residues were solubilized by boiling for 3 minutes in 10 µl of 0.25% (w/v) SDS and 0.1 M ß-mercaptoethanol. No mercaptoethanol was added to the tubes to which O-glycosidase was to be added. 4 mU of endoglycosidase H or 2.5 mU of O-glycosidase (Boehringer Mannheim, Germany) were added in 20 µl of 50 mM sodium phosphate buffer (pH 5.8) containing 0.5 µg of each protease inhibitor and incubated at 37°C for 18 hours. 5 µg ovalbumin, as a positive control for the digestion, was treated similarly. The digestions were stopped by adding 2X Laemmli sample buffer and boiling for 5 minutes. The digestion products were separated by SDS-PAGE, transferred to Immobilon and probed with mAb 4E1. Lanes containing ovalbumin were cut from the rest of the gel before blotting and stained with 0.125% (w/v) Coomassie Brilliant Blue R-250 in 50% (v/v) methanol, 10% (v/v) acetic acid for 1 hour or overnight and destained with 20% (v/v) methanol, 10% (v/v) acetic acid to visualize the protein.

3.2.15 Non-reducing and Two-dimensional SDS-PAGE gels.

25 µg of the proteins obtained from the detergent pellet of Triton X-114 were boiled for 5 minutes in Laemmli sample buffer without reducing agent, loaded into one well of a 1.0 mm thick SDS-PAGE minigel and subjected to electrophoresis at 20 mA constant current for 2.5 hours. For the second dimension gel, the lane was excised, equilibrated in Laemmli sample buffer containing 50 mM dithiothreitol (DTT) for 5 minutes, drained, and loaded horizontally on the top of a second 1.5 mm thick minigel with 100 µl Laemmli sample buffer (+DTT) and run again at 20 mA for 2.5 hours. The gel was blotted onto Immobilon and developed using mAb 4E1 as described above.

3.3 Results

3.3.1 Specificity of monoclonal antibody 4E1

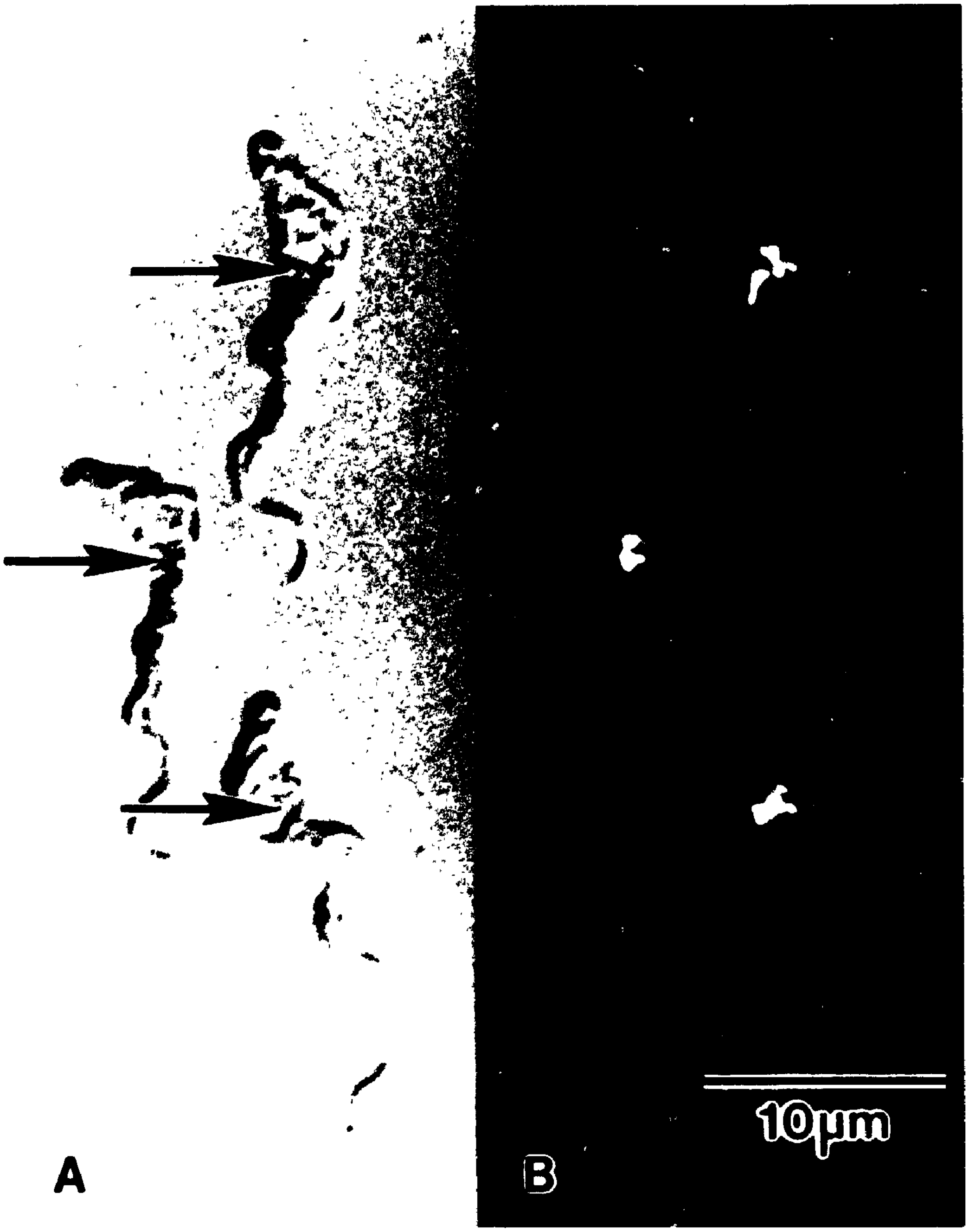
Monoclonal antibodies were raised in Balb/c mice to fixed, 3 day, uncoated, culture forms of *T. vivax* IL 1392. Immunofluorescence on thick cryosections of bloodstream forms of *T. vivax* ILDat 1.2, raised in rats, was carried out in order to localize the reactive antigens as the primary method for screening tissue culture supernatants. One of the monoclonal antibodies, mAb 4E1, labelled internal structures giving a punctate pattern of fluorescence. Unfortunately, it was not possible to determine the intracellular location of this labelling on sections. Labelling of glutaraldehyde fixed Triton X-100 permeabilized bloodstream form *T. vivax* ILDat 1.2 revealed the labelling pattern to be punctate and localized to the posterior region of the trypanosome (Fig. 3.1). The distribution of the label within this region varies depending on the individual cell examined, but the majority of the label is in close proximity to the nucleus. Unpermeabilized trypanosomes show no fluorescence above the background level and no fluorescence was obtained with an irrelevant antibody (mouse monoclonal antibody, IgG_1, directed to *T. brucei* procyclin--a gift from T. W. Pearson) or the FITC-conjugated second antibody alone (not shown). These results suggest that mAb 4E1 recognizes a molecule inside the cell associated with specific structures.

Immunoblots using mAb 4E1 on the separated proteins of whole trypanosome lysates show that the antigen recognized has a relative mobility of 65 kDa and present in all life stages of the parasite (Fig. 3.2 A and B, lanes 1-4). The 55 kDa band seen in Fig. 3.2 B (lanes 1 and 2) was recognized by the ^{125}I-anti mouse second antibody alone (lane 5), suggesting that it is parasite-associated mouse Ig acquired during growth *in vivo*. The 55 kDa band is also detected in the lysate of the uncoated culture forms (Fig. 3.2 B, lane 2) as they were produced by seeding cultures with mouse-derived bloodstream forms and no passages were made. However, the 55 kDa was not detected in the other culture forms, epimastigotes (Fig. 3.2 B, lane 3) and metacyclics (Fig. 3.2 B, lane 4) since it was either degraded or diluted out with subsequent passages. Although it appears that there is relatively more of the gp65 antigen in the epimastigote stage of the parasite (Fig. 3.2 B, lane 3), the relative amount of antigen in each cell type cannot be compared by this method because an equal loading on a protein basis (25 μg protein/lane) probably does not reflect an

Figure 3.1

Immunofluorescence on bloodstream form *T. vivax* ILDat 1.2.

Immunofluorescence using mAb 4E1 and FITC-conjugated anti-mouse second antibody on fixed, Triton-X 100 permeabilized bloodstream forms of *T. vivax* ILDat 1.2, isolated from rats. The same cells are pictured in both panels with (A) being cells photographed under Nomarski optics and (B) is the fluorescence labelling pattern.

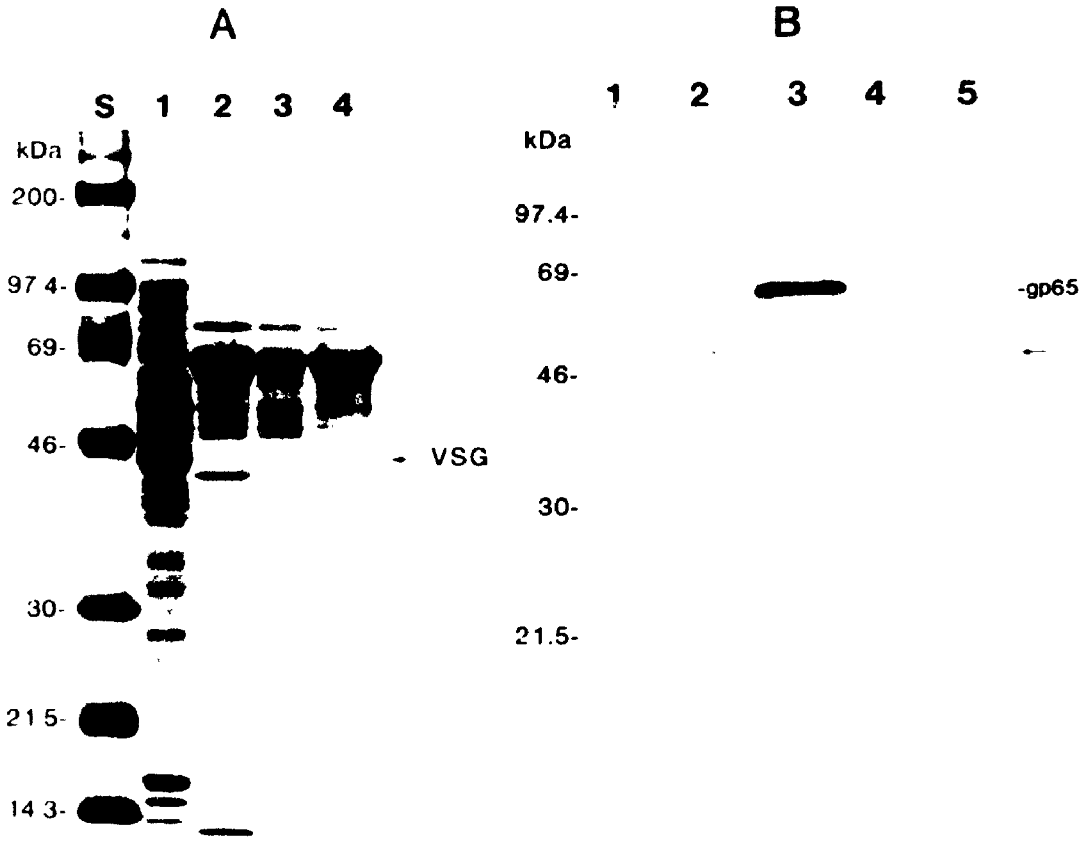


10μm
A
B

Figure 3.2

gp65 is associated with all life cycle stages of *T. vivax*.

Lysates of the different life cycle stages of *T. vivax* ILDat 2.1 were separated on SDS-PAGE gels and either stained with Coomassie blue (A) or blotted onto Immobilon and probed with mAb 4E1 (B). Lanes 1-4 in both A and B contain 25 μg of the cell lysate of bloodstream forms (lane 1), uncoated forms (lane 2), epimastigote forms (lane 3) and metacyclic forms (lane 4). Lane S in (A) contains protein molecular weight markers (10 μl) and lane 5 in (B) contains 25 μg of bloodstream form lysate probed with 2 μCi ^{125}I-anti mouse Ig alone.


A
S
1
2
3
4
kDa
200-
97 4-
69-
46-
30-
21 5-
14 3-
VSG
B
1
2
3
4
5
kDa
97.4-
69-
46-
30-
21.5-
-gp65

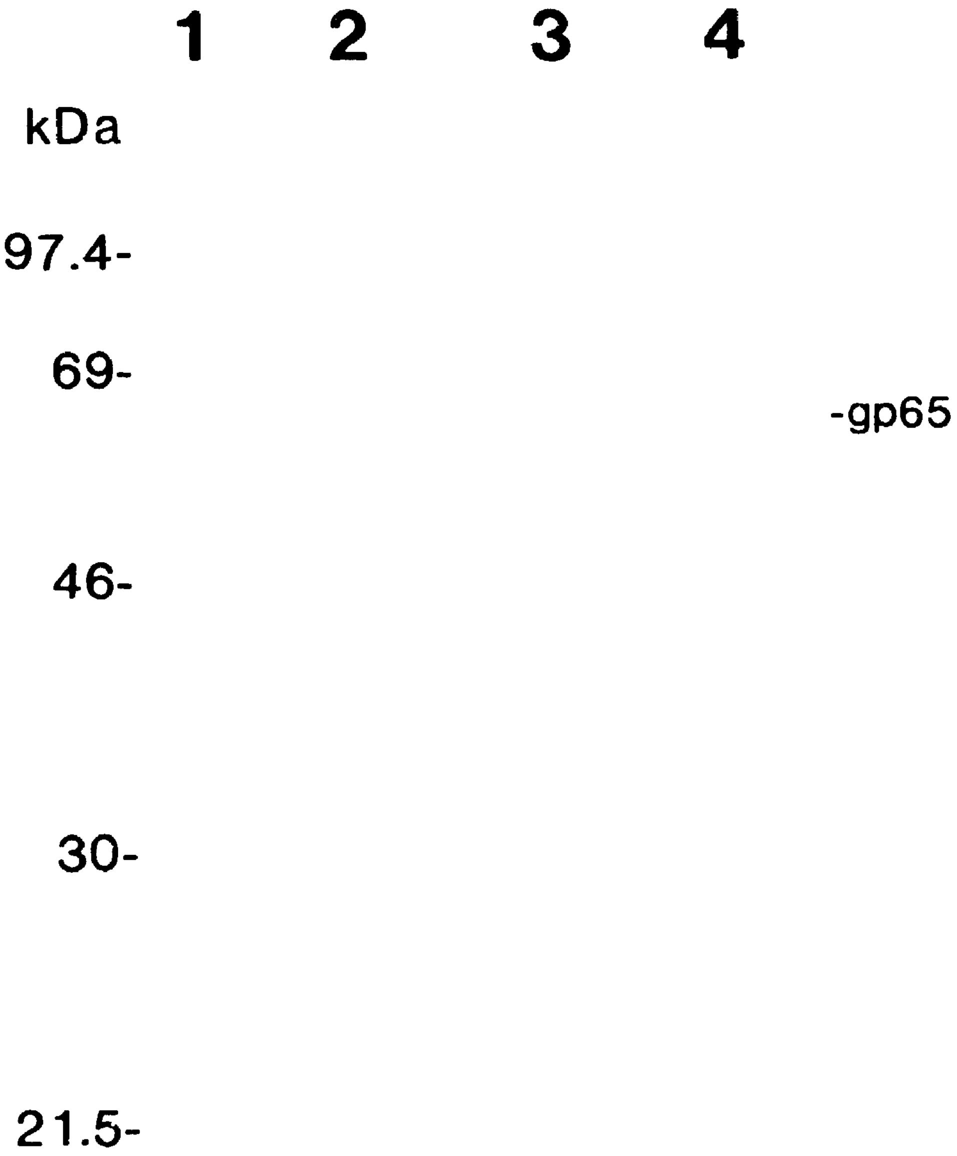
equal cell number loaded per lane due to differences in cell volumes and the amount of protein adsorbed to or ingested by the cells. The Coomassie stained gel of the life cycle stages (Fig. 3.2 A) reveals the position of the VSG in the bloodstream lysate (lane 1), which is apparently absent in the culture forms (lanes 2 and 3) and the metacyclic forms (lane 4). A major band at 69 kDa which is probably bovine serum albumin associated with the culture derived trypanosomes (Fig. 3.2 A, lanes 2-4) Although there may be differences in the amount of antigen expressed in each cell type, these results demonstrate that there is no absolute stage-specific expression of the 65 kDa antigen, as it is present to some degree in each life cycle stage.

T. vivax ILDat 1.2 is a clone derived from a naturally rodent-infective stock, but the vast majority of *T. vivax* isolates are not rodent-infective. Therefore, the reactivity of mAb 4E1 was tested on an immunoblot against other *T. vivax* isolates to ensure that the 65 kDa antigen was present in natural bovine isolates. Fig. 3.3 demonstrates that as well as being present in *T. vivax* ILDat 1.2 from mice (lane 3), it was also present in a lysate of *T. vivax* ILDat 2.1, a clone which was adapted to rodents in the laboratory and isolated from rats (lane 2). It was also present in a lysate of *T. vivax* IL 3067, an isolate from the Kenyan coast district (lane 1) and *T. vivax* Palmira, an isolate from Colombia in South America (lane 4), both of which were grown in calves. The 55 kDa band is only present in the lysate of *T. vivax* ILDat 1.2 harvested from mice. Therefore, the 65 kDa antigen is present in all isolates of *T. vivax* tested, from East and West Africa as well as in a South American isolate which cannot undergo cyclical development in the tsetse fly (Dirie et al., 1992). These results

Figure 3.3

gp65 is present in different geographical isolates of *T. vivax*, grown in different hosts.

Western blot of *T. vivax* lysates from different isolates, probed with mAb 4E1. Lane 1, IL 3067, from Bamburi, Kenya, grown in a calf; lane 2, ILDat 2.1 from Lugala, Uganda, raised in rats; lane 3, ILDat 1.2 from Nigeria, raised in mice; lane 4, an isolate from Palmira, Columbia, raised in a calf. 25 µg total protein in each lane.


1
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kDa
97.4-
69-
-gp65
46-
30-
21.5-

demonstrate that this antigen is not an artifact produced by rodent adaptation since field isolates also have the antigen.

The reactivity of mAb 4E1 is species-specific as demonstrated in Fig. 3.4. This immunoblot contains lysates of different life cycle stages of *T. brucei* (lanes 2, 3 and 8) and *T. congolense* (lanes 4-7), as well as another kinetoplastid, *Leishmania donovani* (lane 9). The only lanes showing immunoreactivity are lanes 1 and 10 which both contain lysates of *T. vivax*. Again, the 55 kDa band corresponding to mouse Ig can be seen in the mouse-derived *T. vivax* (indicated by arrowhead). Therefore, mAb 4E1 reacts with the 65 kDa in a species-specific manner, but whether the antigen is species-specific is not known.

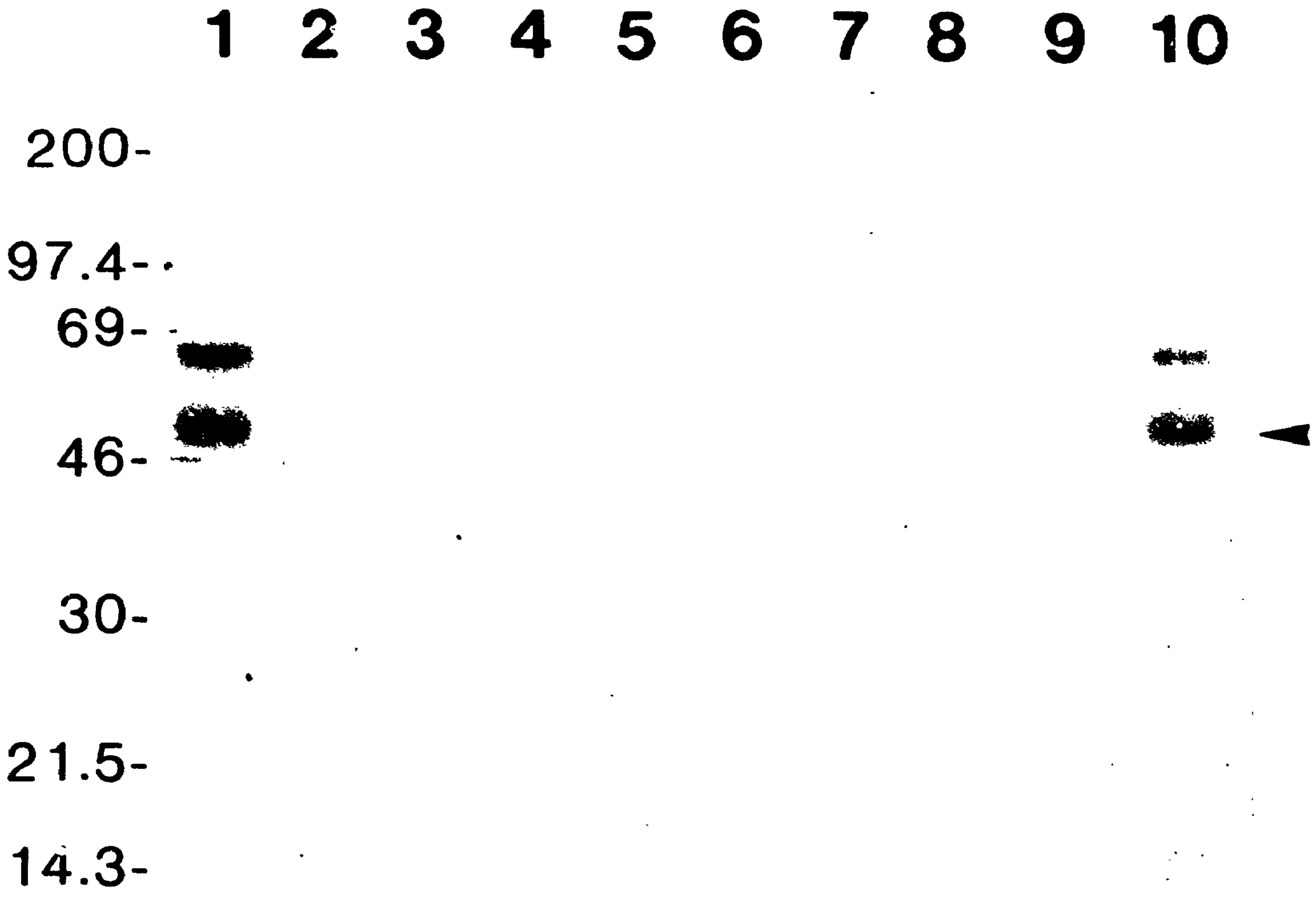
3.3.2 Immunolocalization of the 65 kDa antigen on thin cryosections.

Immunofluorescence using mAb 4E1 on both thick cryosections and whole permeabilized cells demonstrated punctate labelling. A more detailed immunolocalization was performed on thin cryosections of bloodstream form *T. vivax* to better assess which organelles the 65 kDa antigen was associated with in a relatively rapid manner before embarking on biochemical studies. Fig 3.5 demonstrates that the labelling achieved with mAb 4E1 is associated with the periphery of roughly spherical organelles (indicated by arrows) and that some of the labelled organelles are more electron dense than others. No generalized cytoplasmic, surface or nuclear labelling could be seen. Due to the fact that the mAb-labelled organelles were restricted to the posterior region of the cell, as seen in

Figure 3.4

Species-specificity of mAb 4E1.

Whole cell lysates were separated on SDS-PAGE, blotted and probed with mAb 4E1. *T. vivax* ILDat 1.2 bloodstream forms harvested from mice (lanes 1 and 10); *T. brucei* GuTat 3.1 bloodstream forms, rat derived (lane 2); *T. brucei* GuTat 3.1 procyclic forms (lane 3); *T. congolense* bloodstream forms, rat derived (lane 4); *T. congolense* procyclic forms (lane 5); *T. congolense* epimastigote forms (lane 6); *T. congolense* metacyclic forms (lane 7); *T. brucei* ViTat bloodstream culture forms (lane 8); *Leishmania donovani* promastigotes (lane 9). 25 µg protein loaded per lane.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97.4-
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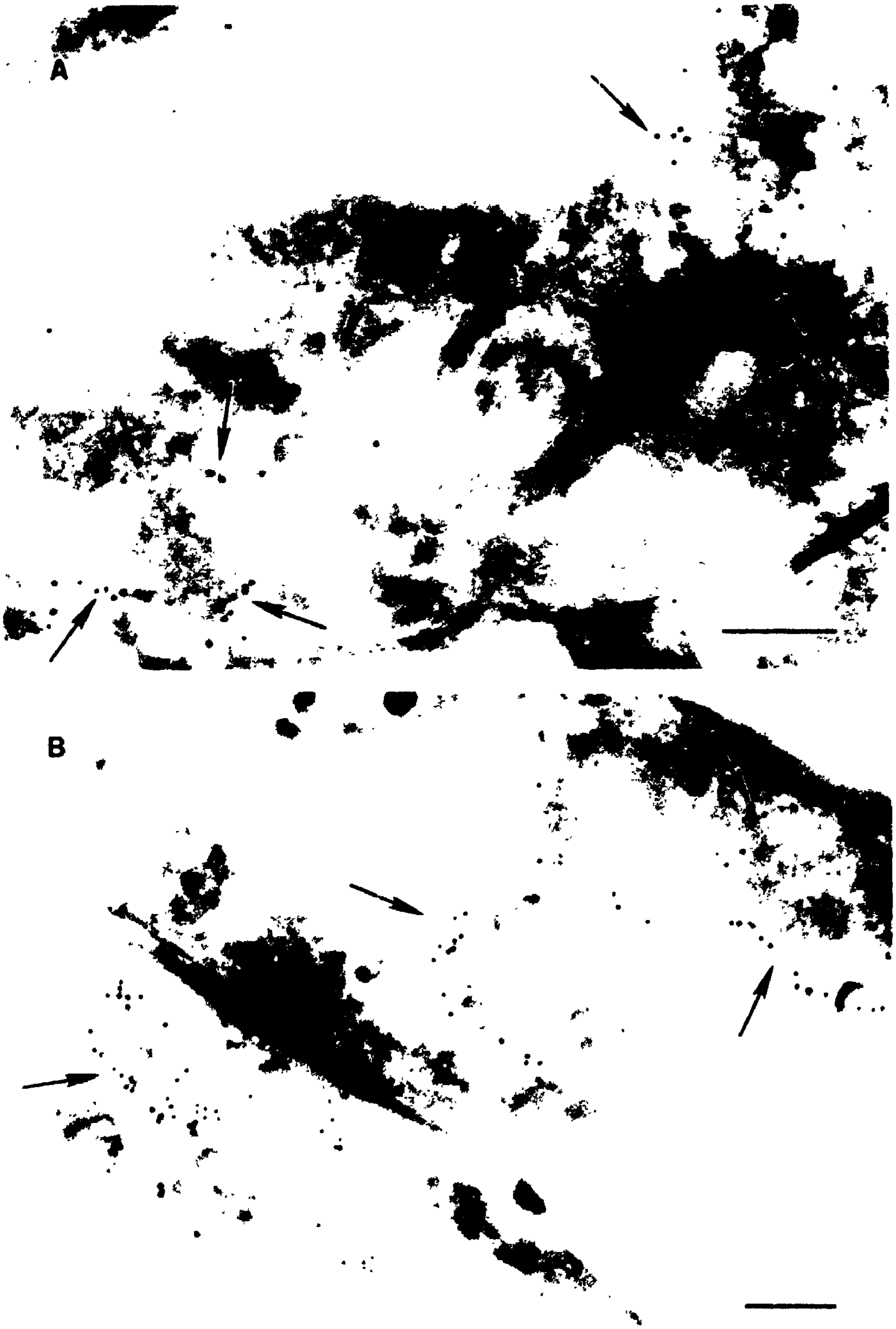
21.5-
14.3-

Figure 3.5

Immunolocalization of gp65 on thin cryosections of *T. vivax* ILDat 2.1.

Ultrathin cryosections of glutaraldehyde-fixed *T. vivax* ILDat 2.1 labelled with mAb 4E1 tissue culture supernatant diluted 1:100. The second antibody label used was a rabbit anti-mouse immunoglobulin which was visualized with 10 nm protein A-gold. Labelling indicated with arrows. Scale bar = 0.25 µm.


A
B

immunofluorescence, it was concluded that these organelles were not glycosomes since they are found throughout the cell.

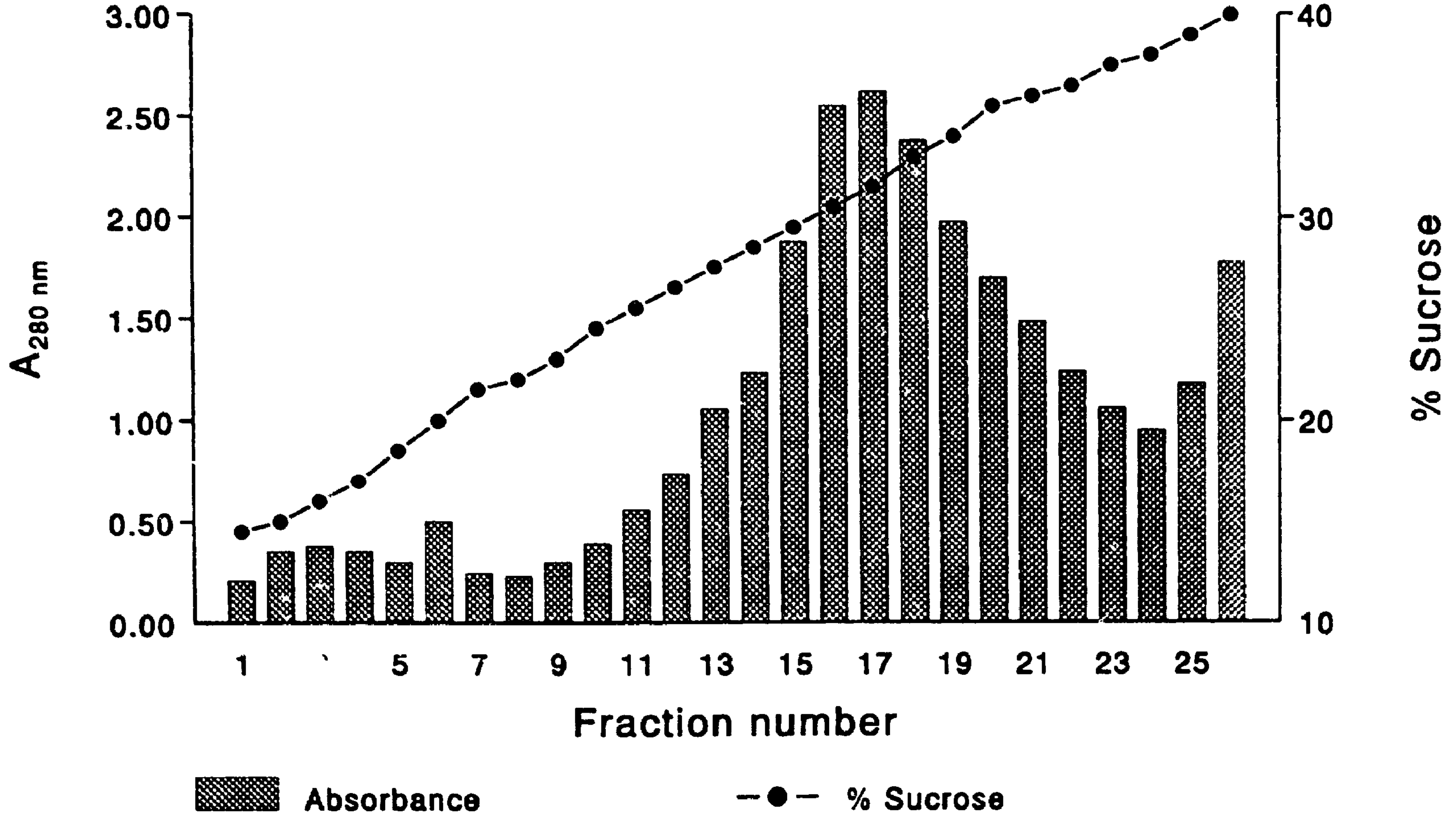
3.3.3 Membrane association of the 65 kDa antigen.

In order to obtain information about the 65 kDa antigen recognized by mAb 4E1, some of its biochemical properties were assessed. In these and all subsequent studies, bloodstream forms of *T. vivax* ILDat 2.1 harvested from rats were used unless otherwise stated.

A) Subcellular fractionation. Disrupted cells were subjected to differential centrifugation. Aliquots from each pellet fraction and the final high speed supernatant (HSS) were electrophoresed, blotted and probed with mAb 4E1 to detect a possible enrichment of the 65 kDa within any fraction. The values of spectrophotometric absorbance ($A_{280\ nm}$) and the concentration of sucrose of each fraction obtained from the sucrose gradient centrifugation are illustrated as a graph (Fig. 3.6) The protein profiles of selected fractions are shown on the Coomassie stained gel (Fig. 3.7 A) A similar gel was electrophoretically transferred to Immobilon and mAb 4E1 used to detect the 65 kDa antigen in each fraction (Fig. 3.7 B). The antigen (gp65) could be detected in all of the membrane fractions with no apparent enrichment within a particular fraction (Fig. 3.7 B, lanes 2-6), but none could be detected in the HSS which represents the fraction of soluble proteins (Fig. 3.7 B, lane 7) When the 120,000*g* pellet was resuspended and centrifuged through a linear sucrose density gradient, the 65 kDa antigen was found in all of the fractions (Fig 3.7 B, lanes 8-18)

Figure 3.6

Protein profile of *T. vivax* membranes subjected to centrifugation on a 15-40% linear sucrose gradient.



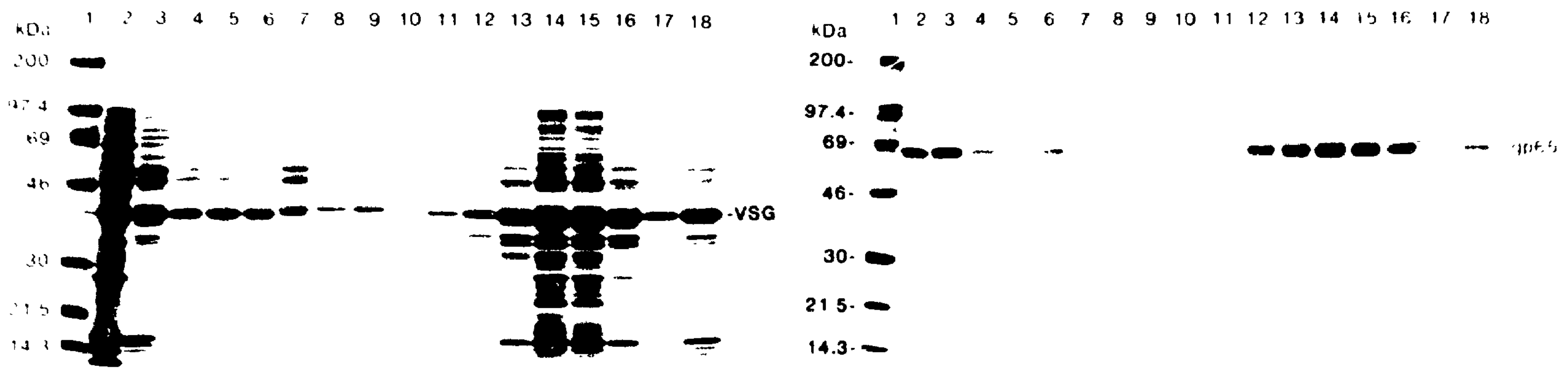
Sucrose Density Gradient
T. vivax membranes

Figure 3.7

gp65 is associated with membrane fractions of *T. vivax* with no apparent enrichment in any fraction.

(A) Coomassie blue stained gel. (B) Western blot of a duplicate gel, probed with mAb 4E1. Lanes 2-7 contain starting material and fractions from the differential centrifugation steps (25 µg protein/lane). Lane 1, 14C-markers (5 µl); lane 2, *T. vivax* ILDat 2.1 bloodstream forms; lane 3, 750g pellet; lane 4, 4,500g pellet; lane 5, 30,000g pellet; lane 6, 120,000g pellet; lane 7, 120,000g supernatant. Lanes 8-18 contain aliquots from selected fractions from the sucrose density gradient (50 µl from each fraction). Lane 8, fraction 2; lane 9, fraction 4; lane 10, fraction 6; lane 11, fraction 9; lane 12, fraction 11; lane 13, fraction 13; lane 14, fraction 16; lane 15, fraction 18; lane 16, fraction 20; lane 17, fraction 24; lane 18, fraction 26.

Subcellular Fractionation of T. vivax ILDat 2.1


kDa
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
200
97.4
69
46
30
21.5
14.3
-VSG
kDa
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
200-
97.4-
69-
46-
30-
21.5-
14.3-
gp65

A

B

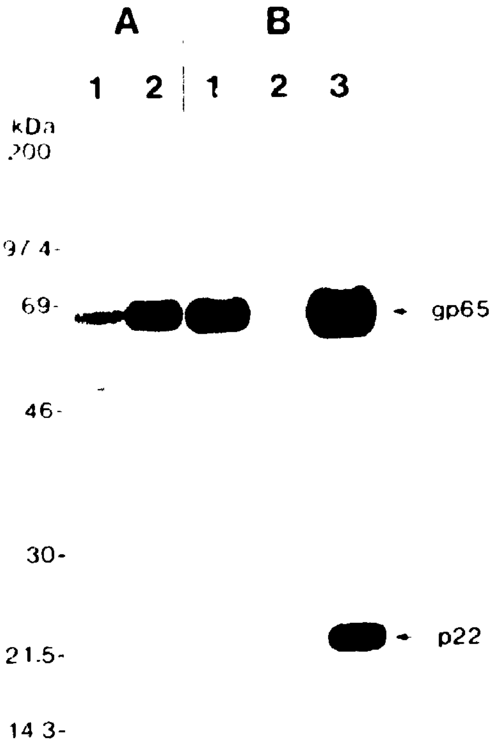
in which the majority of the protein was found (Fig. 3.7 A, lanes 8-18). Therefore, no membrane fraction enriched in the 65 kDa antigen was obtained using this method, consequently, crude membranes were prepared as starting material for enrichment of the 65 kDa molecule in subsequent studies.

B) Na_2CO_3 extraction and Triton X-114 phase separation. Since the 65 kDa antigen was found to be associated with membrane fractions, the nature of this association was investigated. Crude membranes were treated with Na_2CO_3 at alkaline pH to remove both soluble proteins trapped within closed vesicles and peripheral membrane proteins. The 65 kDa antigen was found to be tightly associated with the membrane pellet obtained after sodium carbonate extraction (Fig. 3.8 A, lane 2) and only a small proportion was found in the supernatant fraction (Fig. 3.8 A, lane 1). In a separate experiment, crude membranes were solubilized with Triton X-114 and solubilized proteins were subjected to phase partitioning separation at 37°C. The 65 kDa protein was found to be associated with the detergent phase of Triton X-114 (Fig. 3.8 B, lane 3), which suggests that because of its hydrophobic nature, it is probably an integral membrane protein. In addition to the presence of the 65 kDa antigen in the detergent phase of Triton X-114, another immunoreactive molecule of approximate molecular weight 22 kDa was also highly enriched (Fig. 3.8 B, lane 3). Similar gels to those depicted in Figs. 3.8 (A and B) were Coomassie stained and are shown in Figs. 3.8 (C and D respectively). Coomassie stained gels show that VSG is the major protein band and that it is associated with the membrane pellet after Na_2CO_3

Figure 3.8

gp65 is tightly associated with membranes

Membranes were extracted with Na_2CO_3 (A and C) or solubilized in Triton X-114 and partitioned into different phases (B and D). Centrifugation following Na_2CO_3 treatment resulted in a supernatant fraction (A and C, lane 1) and a pellet fraction (A and C, lane 2). Membrane proteins solubilized with Triton X-114 (B and D, lane 1) were partitioned into an aqueous phase (B and D, lane 2) and a detergent phase (B and D, lane 3). A and B are immunoblots of the described fractions, while C and D are protein profiles stained by Coomassie blue. 25 µg of total protein was loaded in each lane.


A
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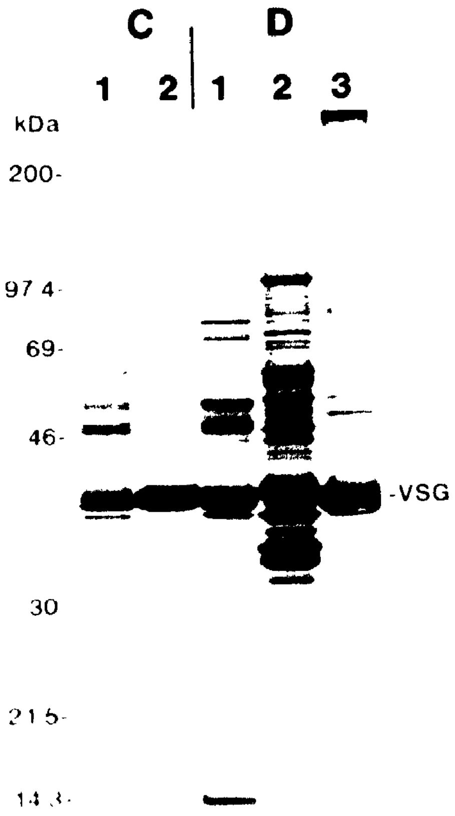
30
21.5
14.3
gp65
p22


C
D
1
2
1
2
3
kDa
200
97.4
69
46
30
21.5
14.3
VSG

extraction (Fig. 3.8 C, lane 2) and the Triton X-114 detergent phase (Fig. 3.8 D, lane 3).

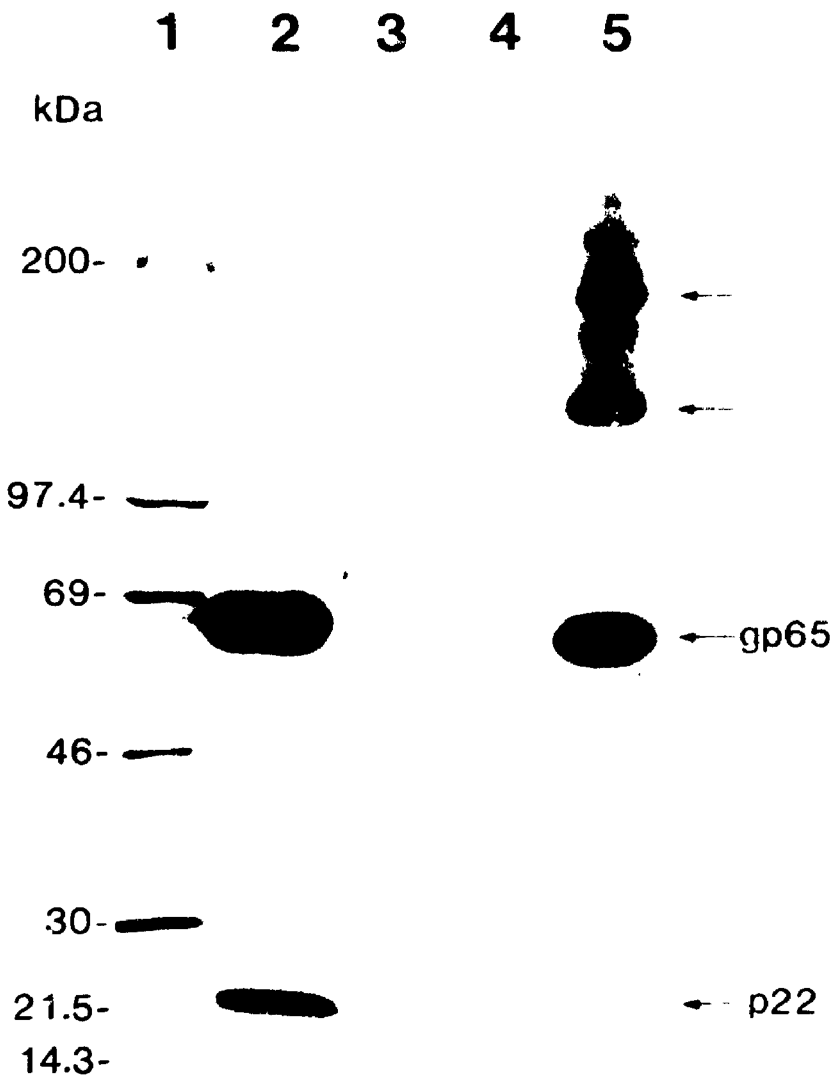
3.3.4 Relationship between the 65 kDa and 22 kDa immunoreactive molecules.

Proteins from the Triton X-114 detergent pellet were electrophoresed under reducing conditions and compared, after immunoblotting, to a similar sample run under non-reducing conditions (Fig. 3.9). Under reducing conditions, both the 65 kDa and 22 kDa antigens were detected (Fig. 3.9, lane 2), but under non-reducing conditions (Fig. 3.9, lane 5), a molecule with an apparent molecular mass of 65 kDa could still be detected, whereas the 22 kDa could not. Additionally, two prominent higher molecular weight bands corresponding to approximate molecular masses of 130 kDa and 190 kDa, were seen in the non-reduced sample (Fig. 3.9, lane 5) These results suggest that the higher molecular weight species are disulfide linked complexes composed of at least one of the immunoreactive species, the 65 kDa or the 22 kDa In order to investigate this possibility, two-dimensional SDS-PAGE gels were run and immunoblotted (Fig. 3.10). For these gels, proteins from the Triton X-114 detergent phase were first electrophoresed under non-reducing conditions, similar to those depicted in Fig. 3.9 (lane 5). The lane was then excised and electrophoresed in a second dimension under reducing conditions (Baskin and Yang, 1982). The high molecular weight complexes seen in the one dimensional non-reduced sample were resolved to 65 kDa and 22 kDa, as was the molecule at the apparent molecular mass of 65 kDa (Fig. 3.10). In addition, the 22 kDa molecule was aligned below the

Figure 3.9

Behaviour of gp65 and p22 under non-reducing conditions

Western blot ofproteins obtained from the Triton X-114 detergent phase, electrophoresed under reducing conditions (lane 2) and non-reducing conditions (lane 5). Lane 1 contains 5 µl of 14C-methylated rainbow markers and lanes 3 and 4 contain Laemmli sample buffer (non-reducing). 25 µg total protein was run in lanes 2 and 5.


1
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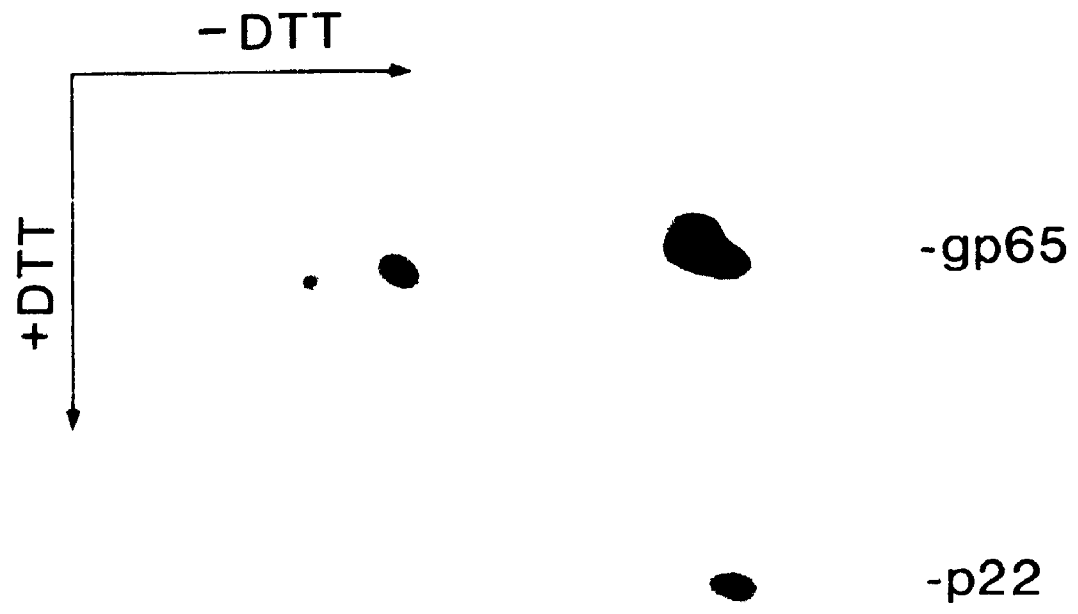
97.4-
69-
46-
30-
21.5-
14.3-
gp65
p22

Figure 3.10

Resolution of gp65 and higher molecular weight complexes to gp65 and p22.

Western blot of Triton X-114 detergent phase proteins run under non-reducing conditions in the first dimension (-DTT) followed by reducing conditions in the second dimension (+DTT). 25 µg protein run in each lane.


-DTT
+DTT
-gp65
-p22

65 kDa molecule in the second dimension on the immunoblot (Fig. 3.10). These results suggest that the 22 kDa molecule is physically associated with gp65 through a disulfide linkage. Thus, p22 may be a fragment of the 65 kDa, generated by hydrolysis and which remains associated with the rest of the molecule due to disulfide linkage(s). In addition, the 65 kDa molecule appears to be able to form oligomeric complexes, possibly homodimers and trimers, but most appears to be in the monomeric state.

3.3.5 The 65 kDa molecule is a glycoprotein containing both N- and O-linked glycans.

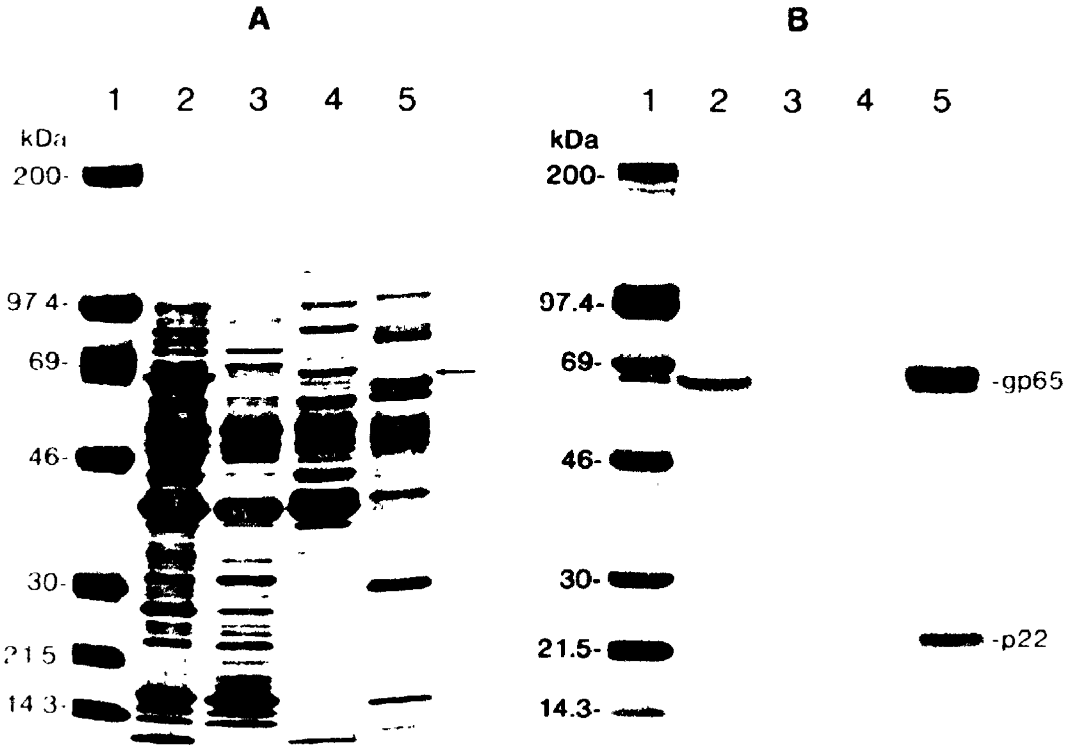
It was possible to obtain a fraction greatly enriched in the 65 kDa antigen by binding solubilized membrane proteins to either Con A-Sepharose or RCA-agarose and eluting with the appropriate specific competing sugar, mannose or galactose, respectively. This indicates that the 65 kDa protein is glycosylated and is referred to hereafter as gp65. The fractions obtained from a typical Con A-Sepharose binding and elution experiment are shown in Fig. 3.11. The protein profile of each fraction was visualized by Coomassie staining (Fig. 3.11 A) and the distribution of gp65 in each fraction was demonstrated by immunoblotting (Fig. 3.11 B). It can be seen that gp65 was totally solubilized with 2% (v/v) NP-40, as there was none detectable in the fraction containing insoluble proteins (Fig. 3.11 B, lane 3). No detectable gp65 was observed in the fraction containing unbound material (Fig 3.11 B, lane 4) although the protein profile of this fraction was more complex (Fig 3.11 A, lane 4) as compared to that of the fraction containing proteins eluted from Con A-Sepharose by α-methyl mannoside (Fig. 3.11 A, lane 5). Therefore, the fraction obtained after specific elution with α-methyl mannoside was greatly enriched in gp65 (Fig. 3.11 B, lane 5). An enrichment for the 22 kDa molecule can also be seen in this fraction. Similar results were obtained when solubilized membrane proteins were bound to RCA-agarose (not shown).

To investigate the nature of the presumptive oligosaccharide linkages of gp65, proteins eluted from RCA-agarose were treated with endoglycosidase H or O-glycosidase (Fig. 3.12). Proteins eluted from Con A-Sepharose were also electrophoresed (lane 1) to demonstrate that gp65 and the 22 kDa protein migrate similarly to proteins eluted from RCA-agarose. Results from treatments of RCA-agarose derived proteins with either glycosidase, showed a reduction in the apparent molecular weight of gp65, but no shift in the relative mobility of the 22 kDa (Fig. 3.12, lanes 3 and 5). Under control conditions, where proteins were incubated in the digestion buffer without glycosidase for the same time period as the samples containing glycosidase, no decrease in the relative mobility of gp65 or and the 22 kDa was observed (Fig. 3.12, lanes 2 and 4). Ovalbumin which contains endoglycosidase H sensitive oligosaccharide linkages was used as a control for enzyme activity (results not shown).

Figure 3.11

Enrichment of gp65 by binding to Concanavalin A-Sepharose.

Solubilized membrane proteins of *T. vivax* ILDat 2.1 bloodstream forms were incubated with Con A-Sepharose. Unbound proteins were washed off and bound proteins eluted with 0.5 M α-methyl mannoside. Fractions separated by SDS-PAGE were stained with Coomassie blue (A) or electroblotted onto Immobilon and probed with mAb 4E1 (B). Lane 1, ^{14}C-methylated rainbow markers (5 μl); lane 2, crude membranes; lane 3, Nonidet P-40 insoluble material; lane 4, fraction of unbound material; and lane 5, proteins eluted from Con A-Sepharose with α-methyl mannoside 25 μg protein loaded in lanes 2-5.


A
1 2 3 4 5
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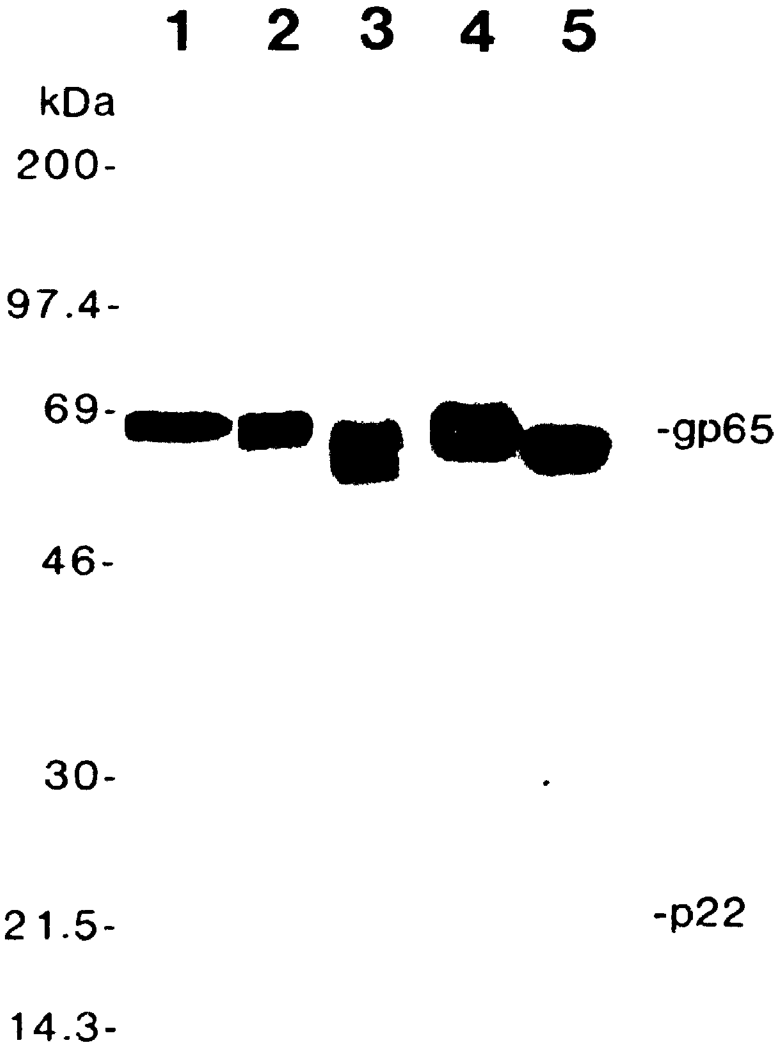
97.4-
69-
46-
30-
21.5
14.3-
B
1 2 3 4 5
kDa
200-
97.4-
69-
46-
30-
21.5-
14.3-
-gp65
-p22

Figure 3.12

Sensitivity of gp65 to glycosidases

Western blot of proteins eluted from RCA-agarose were treated with endoglycosidase H (lane 3), O-glycosidase (lane 5) or were mock treated under the same incubation conditions used for endo H (lane 2) or O-glycosidase (lane 4). Proteins eluted from Con A-Sepharose were run in lane 1. 10 μg protein was used for each treatment.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30-
21.5-
14.3-
-gp65
-p22

3.4 Discussion

Monoclonal antibodies were raised to formalin-fixed three day uncoated culture forms of *T. vivax* for the purpose of identifying invariant membrane proteins. Whole parasites were used for the immunization since not enough cells could be generated to prepare membranes for immunization. In addition, it was hoped that presentation of antigens in this manner may help to direct the antibody response to cell surface proteins. Starting ten days after fusion, tissue culture supernatants were screened by immunofluorescence on thick cryosections of *T. vivax* bloodstream forms. Cryosections were used for screening since no permeabilization step would have to be included to detect internal antigens, thus both surface and internal antigens could potentially be detected on the same section. Although, this method was rapid and was sufficient for an initial screen, since cell sections were used, phase contrast did not yield a clear image of the cell and the localization of the antigen within cells was not easily established. Therefore, selected tissue culture supernatants were re-tested on whole, fixed, permeabilized and non-permeabilized bloodstream forms of *T. vivax*. Using whole cells, binding of mAb 4E1 was clearly localized to the posterior region of the parasite (Fig. 3.1).

On western blots, mAb 4E1 reacted with a 65 kDa antigen (gp65) in all life cycle stages of *T. vivax*. It appeared that the gp65 antigen is more abundant in epimastigote forms of the parasite, and although possible, a quantitative analysis was not carried out. Although an equal amount of each lysate was electrophoresed on a protein basis (25 µg/lane), due to differences in cell size, and the amount of the cell-associated serum proteins, like the 55 kDa mouse Ig, which is associated with the bloodstream and uncoated forms, an equal loading of trypanosome proteins was probably not achieved. Therefore, the apparent greater abundance of gp65 in epimastigotes cannot be confirmed. Although it has been argued that host proteins are not found associated with *T. vivax* by non-specific adsorption (de Gee and Rovis, 1981) it was found in the present study that serum proteins, either from the host or from supplemented cultures, are not readily removed by washing and furthermore, that *T. vivax* is too fragile to withstand repeated washings in protein-free buffers. Of note is the apparent lack of VSG in the lysate of the metacyclic forms (Fig. 3.2 B, lane 4), which is abundant in the bloodstream forms (Fig. 3.2 B, lane 1), as judged by protein staining with Coomassie blue. *T. vivax* metacyclics are infective for mammals and have reaquired a surface coat of VSG (Gardiner et al., 1986a), thus it was expected that VSG would be evident in this sample. Since an obvious VSG band was not detected, it is possible that these metacyclics are preponderently immature forms in which the surface coat is less well developed, or they are contaminated with epimastigotes (Gumm, 1991). Alternatively, it is possible that several VSGs of different molecular weight are being expressed simultaneously.

T. vivax stocks can be divided into two groups, the East African stocks and the West African stocks, based on differences in isoenzyme patterns (Fasogobon et al., 1990). *T. vivax* IL 1392, a West African stock, which was used for immunization can be raised in rodents, however most ruminant-infective stocks of *T. vivax* do not grow in rodents. To ensure that gp65 was present in cattle isolates, western blots of different *T. vivax* isolates were probed with mAb 4E1. gp65 was found to be in all of the *T. vivax* isolates, tested, both East and West African isolates and a non-tsetse transmissible isolate from South America (Dirie et al., 1992) and thus does not appear to be present due to rodent adaptation. In addition, the reactivity of mAb 4E1 was shown to be species-specific as it was not reactive with any life cycle stage of *T. congolense* or the bloodstream or procyclic forms of *T. brucei*. In addition, mAb 4E1 did not react with *Leishmania donovani*, another kinetoplastid protozoon. Since mAb 4E1 works well in immunofluorescence and western blots; it recognizes all *T. vivax* isolates tested, including a geographically isolated *T. vivax* from South America; and does not cross-react with two other trypanosome species which are commonly encountered in livestock infections, it has great potential as a diagnostic reagent for the detection of *T. vivax* in the blood of infected animals.

The use of mAb 4E1 to label thin cryosections of *T. vivax* bloodstream forms revealed a more detailed localization of gp65 to the periphery of vesicular organelles, which suggests that gp65 is associated with the membrane of these organelles. The membrane association of gp65 seen in immunolocalization studies, is supported by biochemical data. Subcellular fractionation of disrupted trypanosomes demonstrated that gp65 was associated with membrane fractions. Furthermore, extraction of membranes with sodium carbonate and the segregation of gp65 into the detergent phase of Triton X-114 suggests that it is an integral membrane protein.

Enrichment of gp65 by Triton X-114 phase partitioning resulted in the co-enrichment of a 22 kDa immunoreactive molecule (p22). The nature of this molecule was investigated since it was important to determine whether it was a distinct cross-reactive protein or a breakdown product of gp65. If p22 was a distinct protein, the immunolocalization experiments would be difficult to interpret. Results from the non-reducing/reducing gel (Fig. 3.9) and the two-dimensional gel (Fig. 3.10) suggest that p22 is associated with gp65 by disulfide linkage. Thus, either p22 is a distinct protein with an epitope similar to that of gp65 or it is a proteolytic fragment of gp65 which bears the epitope and is held to the rest of the molecule via disulfide linkage after the proteolytic event occurs. If p22 is a distinct protein complexed to gp65, non-reducing gels should reveal a band at 87 kDa and higher molecular weight oligomers. Instead, the apparent molecular weight of gp65 under both reducing and non-reducing conditions remains consistent. Therefore, even though there is no hard evidence in favour of either possibility, the more likely explanation is that p22 is a breakdown product of gp65 resulting from a limited proteolytic event.

Under non-reducing conditions, low amounts of higher molecular weight aggregates of gp65 can be detected on immunoblots, which correspond to approximate molecular weights of 130 kDa and 190 kDa. These aggregates can be resolved to gp65 and p22 under reducing conditions thus suggesting that the 130 kDa and 190 kDa are dimers and trimers of the gp65 which are held together by disulfide linkages. However, the majority of the gp65 molecules appear to exist in a monomeric form.

Binding of gp65 to both Con A and RCA indicates the presence of mannose and galactose residues. These carbohydrate linkages can be cleaved by endo H and

O-glycosidase which suggests the presence of both N- and O-linked glycans in the molecule. Although Con A is able to bind N-linked oligosaccharides with a variety of configurations, including the simplest high mannose type, some hybrid type linkages and certain biantennary complex type oligosaccharides, endo H itself is specific for high mannose type linkages. Endo H digestion of gp65 did not result in a distinct lower molecular weight band but a broad smear of lower molecular weight. The same occurred when O-glycosidase was used to digest gp65. Endo H and O-glycosidase were never used simultaneously to determine if a distinct lower molecular weight band could be obtained. Attempts to use N-glycanase were unsuccessful due to protease contamination of the commercial enzyme. Therefore, gp65 appears to be a complex glycoprotein with at least two different types of oligosaccharide linkages and may be heavily glycosylated. The p22 portion of the gp65 was not sensitive to digestion by either endo H or O-glycosidase, therefore, either it has no glycan moieties or they are of the hybrid or complex types that are resistant to cleavage with endo H. Nonetheless, results presented in this chapter provide evidence that gp65 is an integral membrane protein with N- and O-linked glycans. Glycosylation of proteins, especially O-glycosylation, can confer resistance to proteolysis on the molecule (reviewed in Jentoft, 1990). In addition, O-linked glycans occurring in close proximity on the protein can affect the secondary structure by preventing folding of the modified region of the protein, thus giving it a linear structure (Jentoft, 1990).

Preliminary biochemical characterization of the 65 kDa antigen indicates that this molecule is an integral membrane protein which is associated with organelles that are restricted to the posterior portion of the cell. From previous studies on the endocytosis of African trypanosomes, it is known that the endocytic organelles of these parasites are found exclusively within the posterior region of the cell (Langreth and Balber, 1975; Frevert and Reinwald, 1988; Webster and Grab, 1988; Webster, 1989 a, b; Webster and Fish, 1989). In order to further characterize gp65, the possibility that this molecule was localized to an endocytic organelle of *T. vivax* was investigated and is the subject of the next chapter.

CHAPTER 4

Endocytosis by African Trypanosomes and the association of gp65 with the endocytic pathway of *T. vivax*.

4.1 Introduction

4.1.1 Endocytosis in mammalian cells.

Endocytosis is the process by which eukaryotic cells take in exogenous macromolecules and fluid by the invagination and closure of a region of the surface membrane thus forming a new intracellular, membrane-bound organelle (reviewed by Steinman et al., 1983). Endocytosis leads to the internalization of large amounts of plasma membrane and thus the definition should be considered to include the way in which internalized molecules and membrane are sorted within the cell after internalization (Steinman et al., 1983; Besterman and Low, 1983; Helenius et al , 1983). In addition to functioning in the acquisition of nutrient molecules, the endocytic pathway directs the internalization and recycling of physiologically important cell surface receptors, which may be an integrated function of a larger membrane turnover event. The binding of ligands (hormones, growth factors or immunological regulators) to receptors and their internalization can precipitate such activities as cell growth, and secretory or differentiation processes. For example, the internalization of antigens by B-lymphocytes is the first step leading to antigen presentation by these cells (reviewed by Lanzavecchia, 1990).

Molecules are taken into cells both by specific, receptor-mediated endocytosis (Woodman and Warren, 1988; Diaz et al., 1988; Mayorga et al., 1988) and non-specific fluid phase endocytosis (Gruenberg and Howell, 1986; Braell, 1988). During fluid phase endocytosis, or pinocytosis, the concentration of solutes within the endocytic vesicle remains the same as it was in the extracellular medium. Internalization of solutes is achieved simply by "pinching off" an aliquot of the external medium by the invagination of part of the cell surface membrane as a constitutive process. In contrast, receptor-mediated endocytosis occurs following specific interaction of external ligands with surface membrane receptors which leads to the concentration of the complexes within clathrin-coated pits and vesicles (Goldstein et al., 1979). Receptors such as the transferrin receptor and the LDL receptor are endocytosed via clathrin-coated pits regardless of whether they have bound ligand (Anderson et al., 1982; Watts, 1985; Tein and Sussman, 1986), but receptors such as the epidermal growth factor (EGF) receptor require the binding of ligand before endocytosis (Hopkins et al., 1985). Receptors which are internalized via clathrin-coated pits are transmembrane proteins which contain recognition sequences in the cytoplasmic domain that are necessary for high efficiency endocytosis (Collwan et al., 1990; Chen et al., 1990). Several examples are the LDL receptor (Lehrman et al., 1985), the transferrin receptor (Rothenberger et al., 1987; Iacopetta et al., 1988), the immunoglobulin Fc receptor (Mietteinen et al., 1989), and the EGF receptor (Prywes et al., 1986). These recognition sequences function in binding protein adaptor molecules which in turn bind clathrin (for reviews see, Keen, 1990; Pearse and Robinson, 1990). Clathrin assembles as supramolecular cage-like lattices which form the coated pits and coated vesicles evident in the electron microscope (Harrison and Kirchhausen, 1983; Zaremba and Keen, 1983).

At least three different mechanisms for the transfer of molecules from the cell surface to early endosomes can be employed in eukaryotic cells. Clathrin-coated pits have been shown to function in constitutive receptor-mediated endocytosis (Marsh and Helenius, 1980; Pearse and Bretscher, 1981) as well as ligand-induced endocytosis (Hopkins et al., 1985). Caveolae, are non-clathrin-coated membrane invaginations which have been implicated in the receptor-mediated uptake of 5-methyltetrahydrofolic acid by its specific GPI-linked receptor (Rothberg et al., 1990). The cytoplasmic coat of caveolae is distinct from clathrin and has been shown to contain a molecule that is homologous to a 22 kDa substrate for the v-*src* tyrosine kinase (Rothberg et al., 1992). Many ligands and membrane proteins have been shown to enter mammalian and yeast cells following their uptake into non-coated vesicles (for a review, see Sandvig and van Deurs, 1991). Internalization of molecules via non-coated vesicles may be dependent upon intact actin filaments, since disruption of actin filaments in Vero cells by cytochalasin D inhibits uptake via non-coated membranes, but has no effect on the pathway in which clathrin-coated vesicles provide the initial compartment (Sandvig and van Deurs, 1990). In addition, potassium depletion of cells inhibits internalization of molecules by clathrin-coated vesicles but does not inhibit uptake of molecules through non-coated vesicles (Hansen et al., 1991). Therefore, eukaryotic cells have evolved various methods of internalizing exogenous material which appear to operate independently of one another. However, there is evidence that material endocytosed from non-coated vesicles can be co-localized with material originating from clathrin-coated vesicles, thus these two types of endocytic vesicle appear to ultimately contribute to the same internal pathway (Tran et al., 1987).

Newly formed endocytic vesicles fuse rapidly with each other or with pre-existing endosomes to form early endosomes (Helenius et al., 1983). Clathrin must be shed from coated vesicles before fusion of these vesicles can be achieved (Alstiel and Branton, 1983; Rothman and Schmid, 1986). Sorting takes place in the early endosomes and receptors, ligands, plasma membrane and fluid contents are redistributed to specific areas within the cell (Helenius et al, 1983; Simons and Fuller, 1985; Mellman et al., 1986). Endosomal elements isolated from baby hamster kidney cells have been used in fusion studies *in vitro* in which early endosomes exhibit a high degree of specific fusion activity with each other, suggesting that the same may be occuring *in vivo* (Gruenberg et al., 1989). Recently, a family of small GTP-binding proteins has been implicated in different aspects of the regulation of intracellular vesicular membrane traffic (Balch, 1990). One of these proteins, rab5, has been shown to stimulate early endosome fusion *in vitro* (Gorvel et al., 1991), whereas the cell-cycle control protein kinase, cdc2, inhibits endosome fusion (Tuomikoski et al., 1989).

Endocytosed material destined for degradation is transported from early endosomes to late endosomes. Early and late endosomes are functionally and biochemically distinct. Fusion studies have shown that early endosomes have a much higher fusion activity than late endosomes *in vitro* (Gruenberg and Howell, 1986;

1987; Braell, 1987). Although early endosomes are acidic, they are less acidic than late endosomes and lysosomes (Merion et al., 1983; Tansugaran et al., 1984; Murphy et al., 1984; Kielian et al., 1986; Schmid et al., 1988). Early endosomes appear to be involved in the recycling of some receptors (e.g. the transferrin receptor), whereas late endosomes are involved in the delivery of ligands to lysosomes (Schmid et al., 1988) These functional differences are likely to be reflected by the differences seen in the protein composition of their respective membranes (Schmid et al., 1988).

Lysosomes are the final stage in the degradative endocytic pathway (reviewed by Kornfeld and Mellman, 1989). These organelles have a low pH (~5.5) and contain the enzymes necessary to degrade most endocytosed material. Some of these products are transported from the lysosome and used by the cell in biosynthetic pathways. For example, carrier-mediated systems exist in lysosome membranes for the transport of amino acids (Gahl et al., 1982; Pisone et al., 1985; Bernar et al., 1986), sialic acid (Renlund et al., 1986) and cobamalin (Rosenblatt et al., 1986).

The mechanism for the biogenesis of lysosomes is not fully understood (see Kornfeld and Mellman, 1989). After synthesis lysosomal enzymes are processed which includes the phosphorylation of certain mannose residues on high mannose type oligosaccharide side chains. A specific mannose-6-phosphate (M-6-P) receptor in the trans-Golgi network (TGN) binds to these sites and targets the newly synthesized enzymes to lysosomes (Sly and Fischer, 1982; Sahagian, 1984; von Figura and Hasilik, 1986). M-6-P receptors present on the cell surface capture lysosomal enzymes that have been secreted and direct them back to the lysosomes. Thus the formation of a lysosome occurs through the delivery of lysosomal hydrolases to an incoming endocytic compartment, possibly late endosomes. After delivery of lysosomal enzymes to their target organelle by dissociation of the ligand from the receptor due to acidic pH, M-6-P receptors are recycled back to the TGN and the cell surface. In addition to hydrolases, lysosomes contain several membrane glycoproteins (lgp's) which have recently been identified (reviewed in Fukuda, 1991). The function of these molecules is not known, nor is it understood how they are targeted to lysosomal membranes since they lack mannose-6-phosphate but recent evidence suggests that they are sorted intracellularly (Harter and Mellman, 1992) and not routed via the plasma membrane as previously suggested (Lippincott-Schwartz and Fambrough, 1986; Furono et al. 1989; Mane et al., 1989).

As more proteins involved in the endocytic pathway of mammalian cells are found, they will no doubt be useful in the further elucidation of events occurring in the pathway. There are two main schools of thought about how endocytosed material is processed along the degradative pathway. One model, the vesicle transport model, supposes that endocytosed material is delivered to and retrieved from a distinct subset of pre-existing organelles that can be classified as early and late endosomes, pre-lysosomes and lysosomes (reviewed by Griffiths and Gruenberg, 1991). There are many experimental results which can be viewed as evidence for this model. For example, small spherical vesicles with endocytosed material have been observed and are thought to mediate the transport of this material from early endosomes to late endosomes by transport along microtubules. Depolymerization of microtubules with nocodazole arrested the movement of endocytosed material in these small spherical vesicles (Gruenberg et al., 1989; Bomsel et al., 1990). The maturation model, (reviewed by Murphy, 1991), supports the idea that there are no distinct subclasses of vesicles. Endosomes gradually change or mature by successive fusion and fission events whereby essential enzymes are targeted to vesicles, and others are recycled back to the plasma membrane. These events would occur in conjunction with the gradual acidification of the vesicles so that endosomes mature to become lysosomes In the maturation model, transport vesicles can be accounted for since they would be needed to deliver membrane and soluble proteins required for the functional evolution of the degradative pathway.

Whichever model is appropriate, it is clear that the endocytic pathway is a dynamic system. Extensive sorting occurs in the early endosomes, and receptor-ligand complexes are targeted to different intracellular destinations. Some receptors, for example, the LDL receptor, dissociate from their ligands in the early endosome and are re-routed back to the cell surface while the ligand is delivered to the lysosome for degradation. In contrast, the immunoglobulin Fc receptor is delivered to lysosomes and degraded along with its ligand (see Wileman et al., 1985 for a review) and internalized M-6-P receptor ends up in the Golgi apparatus or back at the surface (Brown et al., 1986; Duncan and Kornfeld, 1988). The EGF receptor is usually classified as the type of receptor that is degraded in lysosomes with its ligand (Dunn and Hubbard, 1984; Dunn et al., 1986). However, it has been shown that at low levels of surface receptor occupancy, the receptor is recycled back to the cell surface from endosomes and when greater than fifty percent of the surface receptors are occupied, the complex becomes degraded in lysosomes (Lai et al., 1989).

Many different cultured mammalian cells have been used for studies of endocytosis, but they can be categorized into two basic cell types, polarized and unpolarized. The plasma membrane of polarized epithelial cells, referred to as the apical and basolateral membranes, is segregated into these distinct areas by tight junctions. The apical surface faces the luminal side of the cell while the basal and lateral surfaces face the basal lamina and adjacent cells respectively. Endocytosis can occur from either the apical or the basolateral surfaces of epithelial cells (Anderson and Kaplan, 1983; Steinman et al., 1983). Endocytosed material originating from either the apical or basolateral membranes does not intersect at the level of the early endosome since these vesicles are in close proximity to the membranes from which they originated (Bomsel et al., 1989). However, endocytosed material which was internalized from the apical and basolateral membranes can be found together in the perinuclear late endosomes and lysosomes. This has been substantiated in cell-free assays where apically- and basolaterally-derived endosomes exhibit fusion with each other and these events have been shown to be dependent upon intact microtubules both *in vitro* and *in vivo* (Bomsel et al., 1990). In addition to the mechanisms for sorting of internalized receptors and ligands mentioned above, polarized cells also have an additional pathway, referred to as transcytosis. Transcytosis is the process of internalization of material from one membrane, its transport across the cell and export from the surface of the other membrane. For example, IgG is transported across rat intestinal cells after receptor-mediated endocytosis (Abrahamson and Rodewald, 1981), as is thryoglobulin across thyroid follicle cells (Herzog, 1983).

4.1.2 Endocytosis by African trypanosomes.

Very little is currently known about the processes that occur during endocytosis in African trypanosomes in comparison to the body of knowledge that exists for mammalian cells. The animal-infective forms of trypanosomes live predominantly in the blood of their hosts and are dependent for survival upon the uptake of nutrients from the blood by transport of small molecules and endocytosis. Because trypanosomes are dependent on nutrients acquired through endocytosis, recent interest in this pathway as a potential target for immune intervention has emerged. To better study the mechanism of endocytosis in trypanosomes, it is necessary to identify and characterize specific molecules that are involved in the process.

Endocytosis by trypanosomes has been studied by several investigators and detailed morphological descriptions of the organelles involved have been made (Brown et al., 1965; Langreth and Balber, 1975; Frevert and Reinwald, 1988, Webster and Grab, 1988; Webster, 1989; Webster and Fish, 1989). These studies, using *T. brucei* and *T. congolense*, have shown that trypanosomes are able to take up various particulate and soluble substances from the extracellular medium. Trypanosomes can be compared to polarized mammalian cells since the only site through which these parasites internalize material is via the posteriorly placed flagellar pocket (see 1.5.3 for a description of the flagellar pocket) (Langreth and Balber, 1975; Fairlamb and

Bowman, 1980). It is likely that endocytosis occurs exclusively from the flagellar pocket membrane since it is the only region of the plasma membrane that the pellicular microtubules forming the cytoskeleton are absent (Vickerman, 1969). Uptake occurs through regions of the flagellar pocket membrane which bud off to form vesicles, some of which resemble the clathrin-coated vesicles of mammalian cells morphologically (Pearse and Bretscher, 1981). The material is then transported along a series of intracellular organelles which resemble the endosomes and lysosomes of mammalian cells.

Iron and cholesterol, upon which trypanosomes are dependent for growth, are acquired by these parasites in the forms of transferrin and LDL, respectively. Transferrin and LDL are taken up by trypanosomes via receptor-mediated endocytosis (Coppens et al., 1987) and an LDL-receptor has recently been purified from *T. brucei* (Coppens et al., 1991). In mammalian cells, receptor-mediated endocytosis occurs via clathrin-coated pits resulting in a concentration of the receptor-ligand complex within the coated pits and vesicles. The nature of the molecule(s) which comprise the coat of trypanosome coated vesicles is unknown, and, although it may be a clathrin-like molecule, antibodies to mammalian clathrin do not cross-react with trypanosomal proteins on western blots (Webster and Shapiro, 1990). The trypanosome LDL-receptor has not been characterized well enough to determine whether it has a cytoplasmic domain similar to those found in mammalian cell receptors which have been shown to be important for clathrin binding.

Little is known concerning the mechanism by which endocytosed material is processed by trypanosomes. These parasites possess peptidyl hydrolases some of which are associated with lysosomes (Lonsdale-Eccles and Grab, 1987b). Uptake studies which examined the fate of internalized antibodies specific for the VSG have shown that these antibodies are delivered to an intracellular compartment and degraded (Webster et al., 1990). This degradation can be inhibited by protease inhibitors which inhibit the lysosomal enzymes (Webster et al. 1990). To date, few potential markers for the trypanosomal endocytic pathway have been identified. These markers include an LDL receptor (Coppens et al., 1990), a 77 kDa coated vesicle-associated protein (Webster and Shapiro, 1990), a vacuolar ATPase which may be responsible for the acidification of coated vesicles and early endosomes (W. R. Fish, personal communication), and a *T. congolense*-specific 33 kDa lysosomal cysteine protease (Mbawa et al., 1991). To further study the endocytic pathway of trypanosomes, additional markers of component organelles are needed.

In Chapter 3, the partial characterization of gp65 was reported. In this chapter, a more detailed localization of gp65 within *T. vivax* bloodstream forms is presented. These immunolocalization experiments revealed a very clear association of gp65 with the membranes of tubulo-vesicular structures which are located in the posterior portion of the parasite. Since the trypanosome endocytic pathway is restricted to the posterior region of the cell and the endosomal system of mammalian cells has been described as a network of tubular and vesicular structures, it was of interest to investigate the possible association of gp65 with an endocytic compartment of *T. vivax*. However, since *T. vivax* had not been included in previous studies of endocytosis in trypanosomes (Langreth and Balber, 1975; Frevert and Reinwald, 1988; Webster and Grab, 1988; Webster, 1989a and b; Webster and Fish, 1989), the uptake of soluble and particulate markers of the endocytic pathway by *T. vivax* was examined in this study in order to establish the conditions for endocytosis by this parasite. Subsequently, co-localization studies were carried out using the monoclonal antibody, mAb 4E1, to label thin sections of *T. vivax* bloodstream forms which contained endocytosed bovine serum albumin conjugated to colloidal gold (BSA-gold). The distribution of gp65 in relation to the endocytic pathway in *T. vivax* is discussed.

4.2 Materials and Methods

4.2.1 Preparation of trypanosomes for electron microscopy.

Bloodstream forms of *T. vivax* ILDat 2.1 harvested from rat blood were washed for 10 minutes in PSG/hypoxanthine (pH 7.4), pelleted by centrifugation at 750*g* for 15 minutes, and samples were suspended in fixative containing varying glutaraldehyde concentrations (from 0.5-2%), with 4% (w/v) paraformaldehyde, 0.2% (w/v) picric acid and 0.5 mM $CaCl_2$ in 0.1 M sodium phosphate buffer (pH 7.4). After fixation for 1 hour at room temperature, the trypanosomes were pelleted at 15,000*g* for 3 minutes and the pellet processed by the enhanced membrane contrast method (Berryman and Rodewald, 1990). Graded acetone dehydration was followed by embedding in Lowicryl K_4M resin by the P.L.T. method (Kellenberger et al., 1980; Armbruster et al., 1982). Pellets were polymerized by ultraviolet irradiation (350 nm peak emmission) at -30°C for 12 hours. Other samples of bloodstream form *T. vivax* ILDat 2.1 were fixed in 2% (v/v) glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). After 1 hour of fixation, half of the sample was post-fixed in 1% (w/v) osmium tetroxide. Both preparations were then stained overnight *en bloc* with 1% (w/v) aqueous uranyl acetate, dehydrated in graded acetone and embedded in epon/araldite resin with polymerization at 60°C. In addition to Lowicryl K_4M embedded trypanosomes, those epoxy-embedded samples which were not post-fixed in osmium were used for immunolocalization studies (see below), to give a better indication of the structures being labelled by mAb 4E1.

4.2.2 Immunoelectron microscopy.

Ultrathin sections (40-60 nm thick) of Lowicryl-embedded specimens were collected on copper or nickel grids coated with parlodian/carbon films. All incubations were carried out on 25 µl droplets on parlodian film in a humidified chamber. Single labelling with mAb 4E1 was carried out as follows: sections were preincubated by flotation of the grids on 3% (w/v) BSA in DPBS (pH 7.4) containing 0.5 mM $CaCl_2$ and 1 mM $MgCl_2$ for 30 minutes. Sections were then incubated with mAb 4E1 IgG (diluted 1:4000 in DPBS/BSA) for 1 hour, washed 3 times (5 minutes each) in DPBS/BSA and then labelled with goat anti-mouse 5 nm gold probe (BioCell, Cardiff, UK) diluted 1:10 in DPBS/BSA for 1 hour. Sections were then washed once in DPBS and twice in distilled water (5 minute washes), stained for 10 minutes with 2% (w/v) aqueous osmium tetroxide, stream washed with distilled

H_2O followed by staining for 20 minutes with 2% (w/v) aqueous uranyl acetate. Sections were counterstained with Reynold's lead citrate (Reynolds, 1963) for 10 seconds, washed, dried and examined in an EM10A electron microscope operating at 80 kV (Carl Zeiss, Oberkochen, Germany).

Double labelling with polyclonal rabbit anti-VSG (RαVSG) and mAb 4E1 was performed as follows. After preincubation with DPBS-BSA, sections were incubated in RαVSG (diluted 1:1000 in DPBS/BSA) for 1 hour, washed 3 times (5 minutes each) in DPBS/BSA, then labelled with 15 nm protein A-gold (prepared as described in section 3.2.6) and washed as above. This was followed by incubation with a 1:100 dilution of normal rabbit serum for 30 minutes to block any free protein A sites on the 15 nm probe. Sections were then similarly washed 3 times in DPBS/BSA, incubated with mAb 4E1 and the single labelling procedure followed.

4.2.3 Endocytosis by *T. vivax.*

T. vivax ILDat 2.1 bloodstream forms, isolated from rat blood, were suspended in RPMI-1640 containing 0.1 mM hypoxanthine and checked for viability, as judged by motility. The concentration of 5 nm and 10 nm BSA-gold was standardized to a solution absorbance of 1.0 at 525 nm and trypanosomes were incubated at 37°C in suspensions containing either probe separately, or the probes mixed in equal volumes, at 2 x 10^7 cells/ml. Incubations were carried out for a maximum of 120 minutes, a period in which the trypanosomes maintained their viability. A 500 μl aliquot of cells was taken after 15 seconds, and at various intervals thereafter, was added to 500 μl of double strength fixative and then incubated for 1 hour at room temperature. Cells were then processed as previously described into Lowicryl K_4M or epoxy resins. Cells which had endocytosed 10 nm BSA-gold were used in single and double labelling experiments using mAb 4E1 and RαVSG as previously described.

For uptake of horseradish peroxidase (HRP) by *T. vivax*, the same procedure was followed except that cells were incubated in RPMI containing 0.1 mM hypoxanthine and 5 mg/ml HRP (Sigma) and fixed at various times of incubation. Fixed cells were pelleted at 10,000*g* for 3 minutes and washed 3 times in 0.1 M sodium phosphate buffer (pH 7.4). Diaminobenzidine (DAB) was added at a concentration of 1 mg/ml in 50 mM Tris (pH 7.6) and incubated for 5 minutes. The cells were pelleted and washed once in phosphate buffer then incubated in 500 μl DAB solution containing 0.01% H_2O_2 for 10 minutes, then washed 3 times in phosphate buffer and processed into epoxy resin.

4.3 Results

4.3.1 Intracellular localization of gp65.

A more detailed localization of gp65 was obtained using mAb 4E1 for immunolabelling of thin sections of glutaraldehyde-fixed bloodstream form trypanosomes embedded in either Lowicryl K_4M or epon resin, than was obtained using cryosections (Fig. 3.5). Ultrathin sections of Lowicryl (Fig. 4.1, A and C) and Epon (Fig. 4.1, B and D) embedded cells were used for immunolabelling in which it can be seen that the mAb 4E1 label is specifically associated with tubules and vesicles. Although the degree of labelling achieved on Lowicryl K_4M sections was much greater, membrane preservation and visualization of the specific structures labelled with mAb 4E1 was better with epoxy sections. Therefore, epoxy-embedded trypanosomes were used here for the purpose of more clearly illustrating the membrane association of the label on what appears to be vesicular structures (Fig. 4.1 B) and tubular profiles (Fig. 4.1 D) structures. No labelling was observed on the surface membrane, flagellar pocket, coated vesicles, Golgi apparatus, endoplasmic reticulum or nucleus. Furthermore, no labelling was observed in control experiments using normal mouse serum or the second antibody gold probe alone (not shown).

4.3.2 Endocytosis of BSA-gold and HRP by *T. vivax.*

Results of the immunolocalization studies demonstrated that gp65 is localized at the membrane of vesicles and tubules in the posterior portion of bloodstream form trypanosomes. Since the organelles involved in endocytosis are located exclusively within the posterior region of *T. brucei* and *T. congolense* (Langreth and Balber, 1975; Frevert and Reinwald, 1988; Webster and Grab, 1988), the possible involvement of the mAb 4E1-labelled organelles in endocytosis was investigated. Previous studies of endocytosis in trypanosomes have been carried out with *T. brucei* and *T. congolense* (Langreth and Balber, 1975; Webster and Grab, 1988; Webster, 1989; Webster and Fish, 1989). However, comparable studies of endocytosis with the highly motile and more fragile *T. vivax* required establishment of conditions appropriate for this organism. Initial experiments examined uptake of 5 nm and 10 nm BSA-gold probes.

A time course study was performed to assess the kinetics of BSA-gold uptake, the relative position of the two probes within the cell at each time point, and parasite viability during the incubation period. This experiment revealed that in *T. vivax*, both 5 nm and 10 nm BSA-gold probes are rapidly endocytosed and retained in similar organelles (not shown) and during the incubation time, up to 120 minutes, cells remained viable as judged by motility. Furthermore, when combined in a single incubation the two probes were always co-localized (not shown). Thus, for better visualization, all further experiments were performed using 10 nm BSA-gold (Fig. 4.2).

In endocytosis experiments, BSA-gold particles were present in the flagellar pocket and coated vesicles after 15 seconds (Fig. 4.2 A), which was the minimum time required to mix the trypanosomes with the BSA-gold suspension and remove an aliquot for fixation. Incubation of cells in BSA-gold for between 5 and 30 minutes resulted in the accumulation of endocytosed marker within many internal tubular and vesicular organelles. As incubation time increased, BSA-gold was seen within organelles further from the flagellar pocket and closer to the nucleus, although small amounts of BSA-gold were observed in the flagellar pocket and coated vesicles at the 5 and 15 minute time points (Fig. 4.2, B and C). After 30 minutes the BSA-gold marker appeared to be aggregated within vesicles in close proximity to the nucleus(Fig. 4.2 D), which may be lysosomes. No further movement of endocytosed BSA-gold could be discerned at times up to 2 hours (not shown).

Figure 4.1

Immunolocalization of gp65 on ultrathin sections of Lowicryl-embedded and epoxy-embedded *T. vivax* ILDat 2.1 bloodstream forms using mAb 4E1.

Bloodstream form trypanosomes were fixed with 2% glutaraldehyde and embedded either in Lowicryl K_4M (A and C), or Epon (B and D). Sections were labelled using mAb 4E1 at a dilution of 1:4000 for Lowicryl sections and 1:500 for epoxy sections. Goat anti-mouse 5 nm gold probe was used to visualize mAb 4E1. gp65 is localized to vesicles (v) and tubules (t). Scale bar = 0.25 μm.

A
v
B
v
C
D

Figure 4.2

Endocytosis of BSA-gold by *T. vivax* ILDat 2.1 bloodstream forms.

Sections of Lowicryl-embedded trypanosomes which had been incubated in medium containing 10 nm BSA-gold for 15 seconds (A), 5 minutes (B), 15 minutes (C), and 30 minutes (D). BSA-gold can be seen in the flagellar pocket (fp), coated vesicles (cv) and many internal structures. Nucleus (n). Scale bar = 0.5 µm.

A
fp
fp
cv
fp
cv
B
C
cv
n
fp
D
n
fp

The uptake by *T. vivax* of horseradish peroxidase, a soluble fluid-phase marker for endocytosis, was also investigated to determine if similar intracellular structures as those observed in *T. brucei* and *T. congolense* were involved. HRP, as visualized by the DAB reaction product, can be seen associated with the surface of the cells (s), around the flagellum (f), lining the flagellar pocket (fp), and in a small vesicle that had presumably budded off the flagellar pocket membrane within fifteen seconds of addition of exogenous label (Fig. 4.3 A). At the 30 minute time point (Fig. 4.3 B), HRP can be seen in many tubular and vesicular structures within the cell, including some stacks of the Golgi apparatus (insert).

4.3.3 Co-localization of mAb 4E1 with endocytosed BSA-gold in *T. vivax*.

Since endocytosed BSA-gold occupied different organelles of the cell with increasing times of incubation, 15 seconds to 30 minutes, these time points were used for the co-localization of BSA-gold and mAb 4E1. Fig. 4.4 (A-D) shows Lowicryl sections of *T. vivax* fixed after incubation with 10 nm BSA-gold for 15 seconds, 5, 15, and 30 minutes, respectively. Immunolocalization with mAb 4E1 shows that, after incubation of trypanosomes in BSA-gold for 15 seconds (Fig. 4.4 A), the membrane bound compartments labelled with the antibody do not contain the endocytosed marker. However, varying degrees of co-localization of the mAb 4E1 with vesicles containing endocytosed BSA-gold are seen at the 5 to 30 minutes time points

Figure 4.3

Uptake of horseradish peroxidase by *T. vivax* ILDat 2.1 bloodstream forms.

Epon sections of bloodstream form trypanosomes that had been incubated in 5 mg/ml HRP for 15 seconds (A) or 30 minutes (B) before fixation. Diaminobenzidine and H_2O_2 were used to produce an electron dense reaction product in the region of the cell that HRP was present before embedding in epoxy resin. HRP can be seen on the cell surface (s), lining the flagellar pocket (fp), on the flagella (f), and in cisternae of the Golgi apparatus (insert; g) where the arrow heads indicate the *cis* Golgi. Scale bar = 0.25 µm for A and insert; 0.5 µm for B.

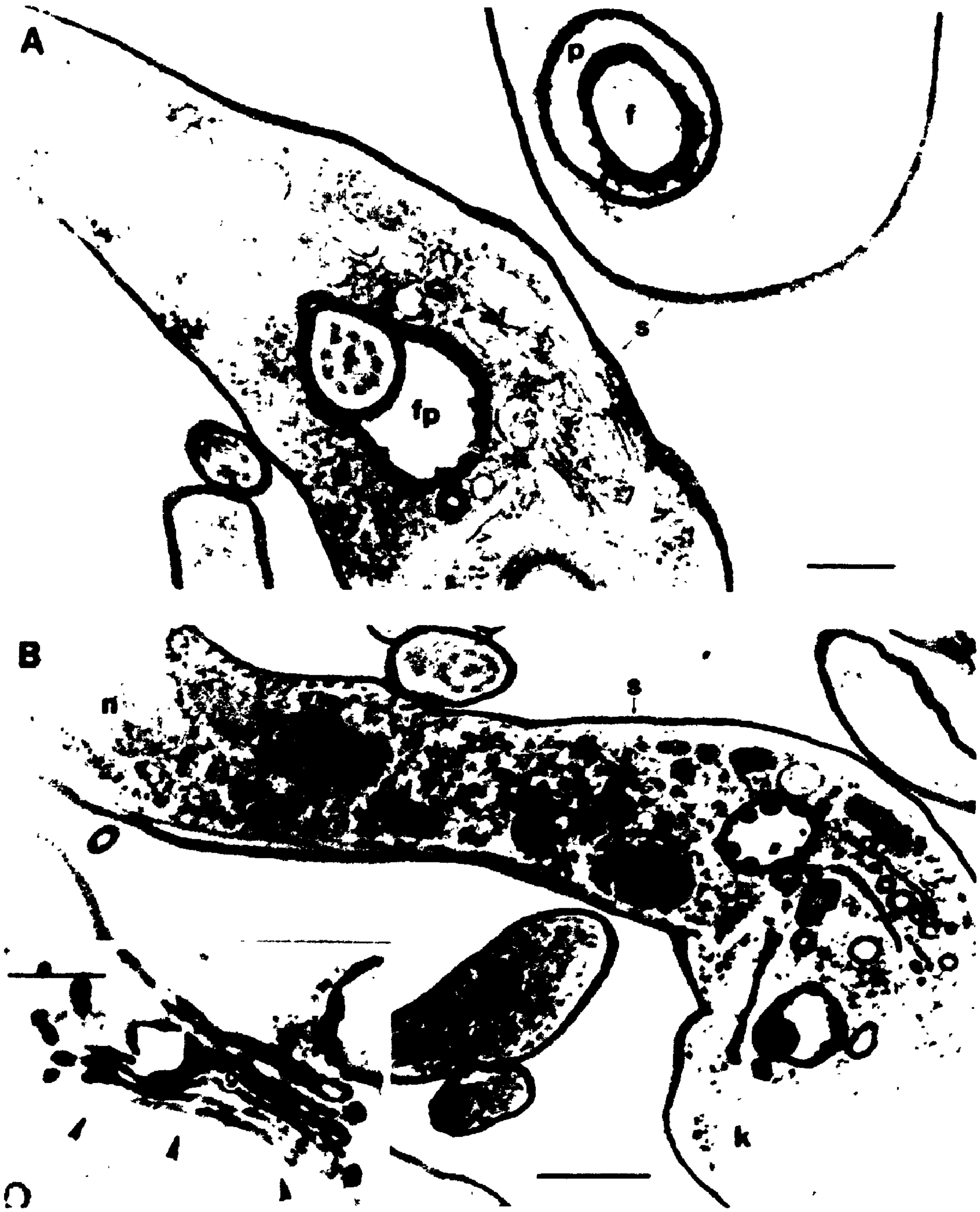
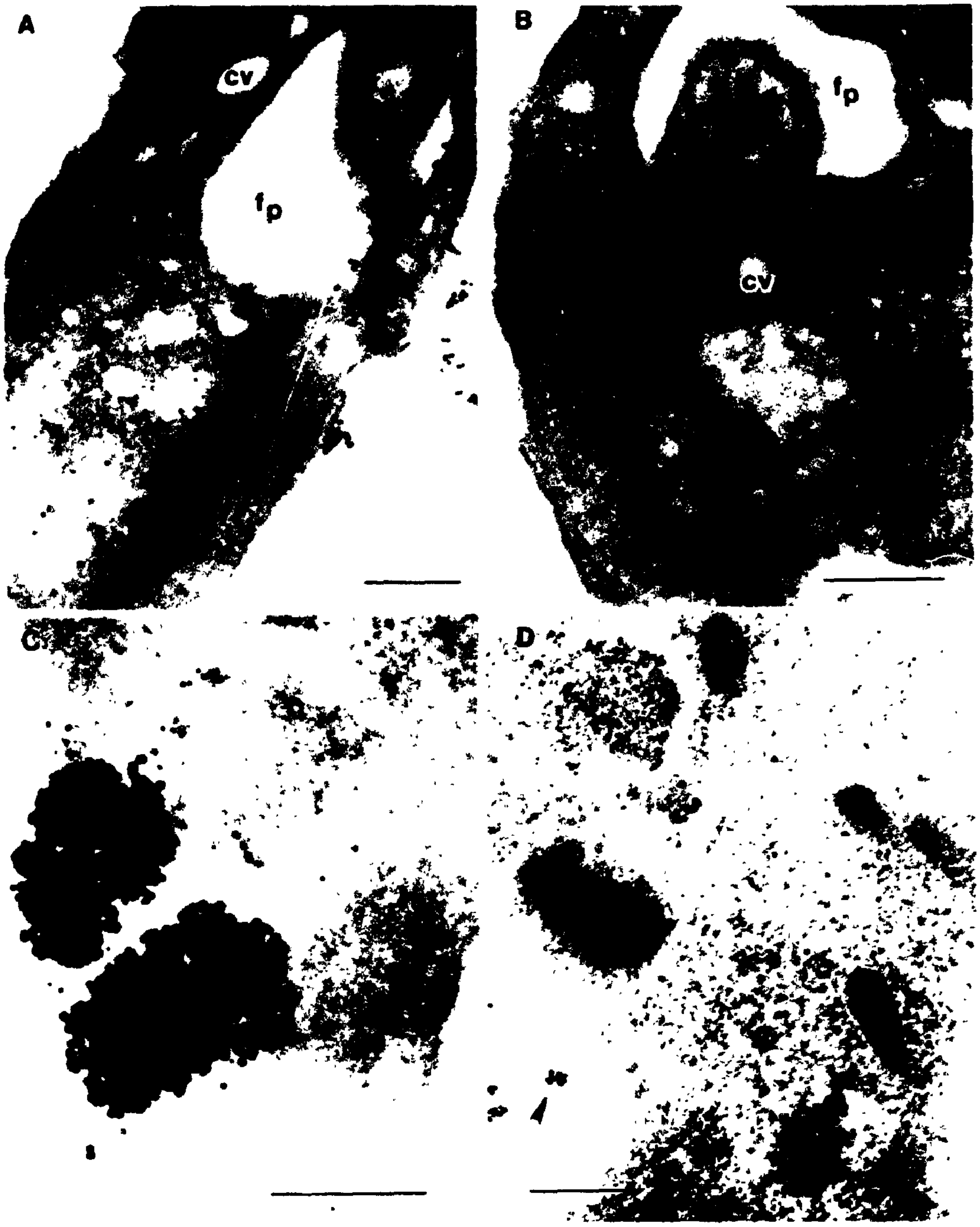

A
p
f
s
fp
B
n
s
k
C

Figure 4.4

Co-localization of mAb 4E1 with endocytosed BSA-gold in *T. vivax* bloodstream forms.

mAb 4E1-labelled sections of Lowicryl K_4M-embedded bloodstream form *T. vivax* which had been incubated in medium containing BSA-gold for 15 seconds (A), 5 minutes (B), 15 minutes (C), and 30 minutes (D). BSA-gold probe is 10 nm and a 5 nm goat anti-mouse gold probe was used to detect mAb 4E1. Flagellar pocket (fp); coated vesicle (cv). Scale bar = 0.25 µm.


A
cv
fp
B
fp
cv
C
D

(Fig. 4.4, B-D). The endocytosed marker, BSA-gold, and the mAb-labelled vesicles show the greatest degree of co-localization after incubation of the parasites in BSA-gold for 5 minutes (Fig. 4.4 B). However, not all vesicles which labelled with mAb 4E1 contained endocytosed BSA-gold, even at the 5 minute time point. The degree of co-localization of endocytosed BSA-gold with the mAb 4E1 label progressively decreases in cells fixed after 15 and 30 minutes of incubation with BSA-gold (Fig. 4.4, C-D).

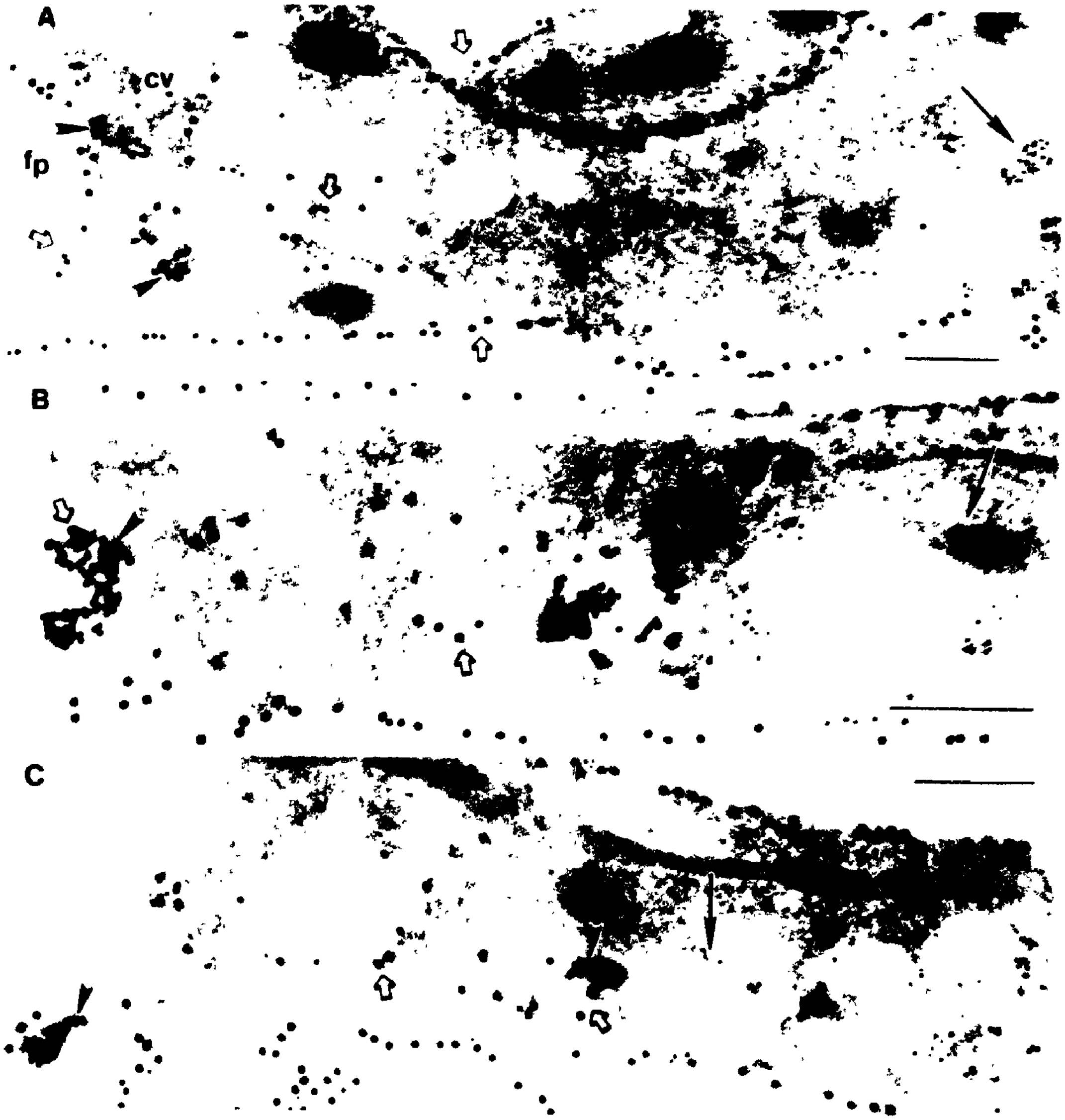
4.3.4 Immunolocalization of mAb 4E1 and RαVSG on sections of *T. vivax* containing endocytosed BSA-gold.

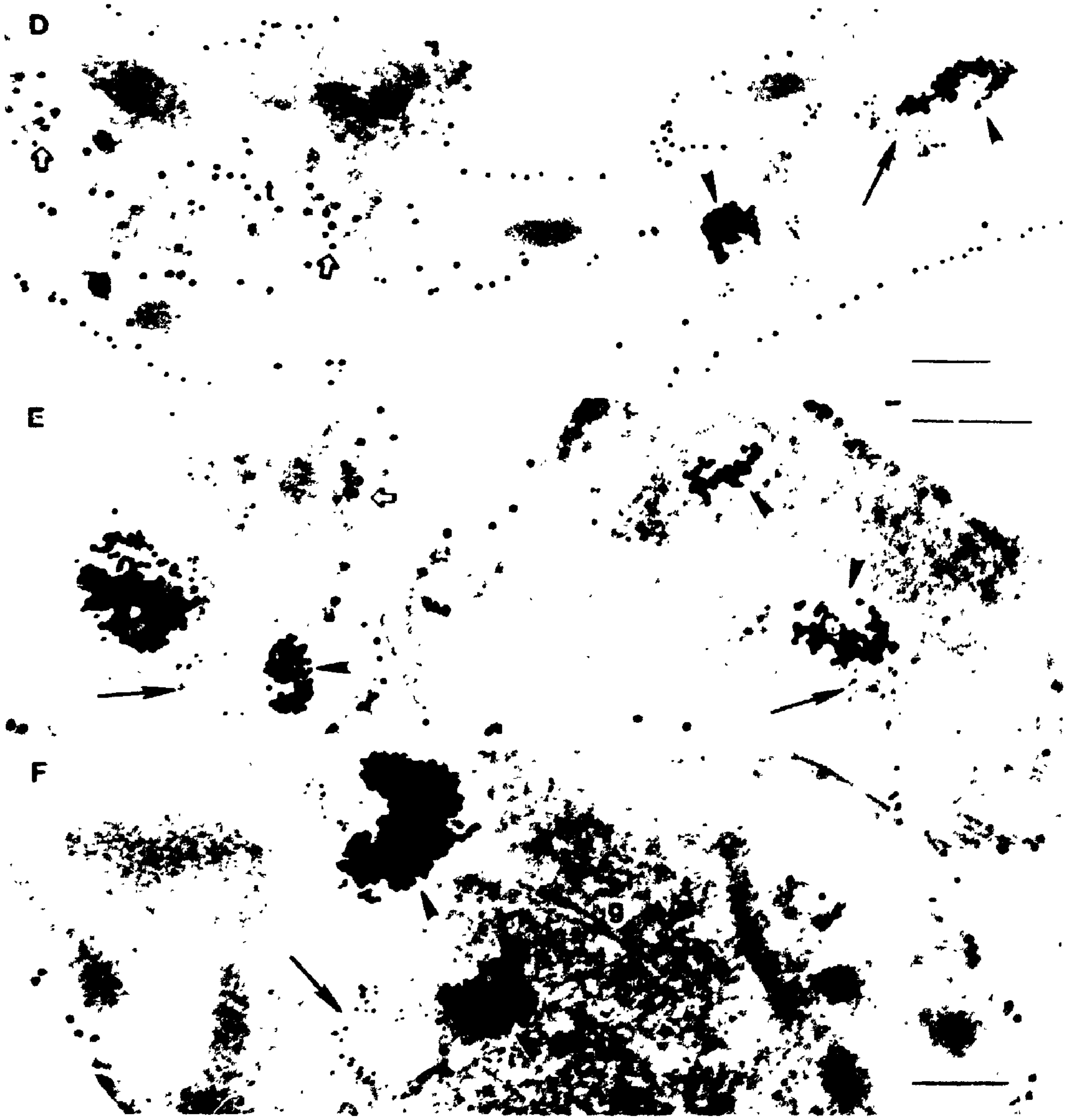
The distributions of mAb 4E1 and RαVSG labels were compared to that of endocytosed BSA-gold at different time points. After 1 minute, both VSG and BSA-gold appear to be internalized via the coated vesicles which have budded from the flagellar pocket membrane (Fig. 4.5 A). In agreement with previous results (Figs. 4.2 and 4.4), BSA-gold is rapidly internalized and can be seen inside the cell, close to the flagellar pocket, after 1 minute of incubation (Fig. 4.5 A). RαVSG label is also found extensively on the surface of the cells, within many vesicles (Fig. 4.5, A-D), and tubules (Fig. 4.5 D), as well as in the Golgi (Fig. 4.5 F). Vesicles labelled with mAb 4E1 are generally further from the flagellar pocket than the RαVSG-labelled structures; thus the two labels only co-localize to a small extent. As already shown (Fig. 4.2), endocytosed BSA-gold appears to progress from the point of entry into the cell, the flagellar pocket, and concentrate in "lysosome-like" organelles near the nucleus by 15-30 minutes. At 1, 2 and 3 minutes, some of the endocytosed BSA-gold co-localizes with the RαVSG label (Fig. 4.5, A-C) although most of the two labels are found in distinct structures. By 3 minutes (Fig. 4.5 C), some of the endocytosed BSA-gold is co-localized with the mAb 4E1 and by 4 and 5 minutes (Fig. 4.5, D and E), this association is more pronounced. After 5 minutes, very little BSA-gold is observed within the region of the cell predominantly labelled by RαVSG. At 15 minutes (Fig. 4.5 F) some of the BSA-gold is co-localized with mAb 4E1, but most is distinct, as observed above (Fig. 4.4 D).

Figure 4.5

Immunolocalization of gp65 and VSG in bloodstream forms of *T. vivax* ILDat 2.1 that had endocytosed BSA-gold.

Sections of Lowicryl K_4M embedded cells that had been incubated in medium containing 10 nm BSA-gold (arrowheads) for 1 minute (A), 2 minutes (B), 3 minutes (C), 4 minutes (D), 5 minutes (E) and 15 minutes (F). Immunolocalization of gp65 using mAb 4E1 (5 nm gold probe; arrows) and VSG using a polyclonal rabbit antiserum (RαVSG) (15 nm gold probe; open arrows). Flagellar pocket (fp); coated vesicle (cv); Golgi apparatus (g). Scale bar = 0.25 μm.


A
cv
fp
B
C


D
t
E
F
g

4.4 Discussion

The ultimate aim of the localization, endocytosis and co-localization studies presented in this chapter, was to gain insight into the nature of the organelle(s) with which gp65 was associated. In doing this, it was hoped that the possible function of gp65 in the cell would become evident. By using mAb 4E1 in immunofluorescence, gp65 was localized to the posterior region of the cell, between the flagellar pocket and the nucleus (Fig 3.1). More detailed immunolocalization, using mAb 4E1 to label thin cryosections of bloodstream form *T. vivax* (Fig. 3.5), indicated that gp65 was most probably vesicle-associated and that it was located at the periphery of these vesicles. The cryosectioning technique was the method of choice for immunolocalization studies, since it generally better preserves the antigenicity of the sample because the only potentially denaturing step is the initial aldehyde fixation (Griffiths et al., 1984). This technique has worked very well for a variety of antigens and cell types (Griffiths et al., 1983; 1988; Brands et al., 1983; Boonstra et al., 1985; Christensen et al., 1985; Webster et al., 1985), including *T. brucei* and *T. congolense* (Webster, 1989b). However, the ultrastructural preservation of internal membranes of *T. vivax* was fairly poor using this technique, even though the level of antibody labelling was excellent (Fig. 3.5). Since subsequent experiments, involving endocytosis and co-localization of mAb 4E1 with endocytosed material, required high resolution of labelled structures, a fixation/embedding method was sought which would give a better balance between ultrastructural preservation and antibody labelling.

Lowicryl K_4M was used to embed *T. vivax* bloodstream forms which had been fixed with various concentrations of glutaraldehyde, up to 2%. Using this method, better ultrastructural preservation was obtained. In addition good antibody labelling was achieved, even with sections of cells which had been fixed with 2% glutaraldehyde. Of the embedding techniques used, epoxy-embedded *T. vivax* had the best morphology (for an example, see Fig. 1.2). Therefore, an attempt was made to carry out immunolabelling on thin sections of these cells. No labelling was obtained on sections of cells that had been post-fixed with osmium tetroxide. However, detectable labelling was achieved on sections of Epon-embedded cells where the osmium post-fixation step was omitted. Samples of mAb 4E1-labelled sections, of both Lowicryl- or epoxy-embedded cells, are shown in Fig. 4.1. Here, the membrane-association of the mAb 4E1 label was most clearly shown in the Epon sections (Fig. 4.1, B and D), but the level of labelling achieved was much greater on sections of Lowicryl-embedded *T. vivax* (Fig. 4.1, A and C). The improved ultrastructural preservation obtained with these alternative embedding methods, allowed the mAb 4E1-labelled structures to be more clearly resolved as the membranes of tubular and vesicular structures. Serial sectioning was not done, thus it is not known to what extent the tubulo-vesicular structures were interconnected.

The endocytic organelles of *T. brucei* have been shown to exist exclusively within the posterior portion of the cell (Langreth and Balber, 1975; Frevert and Reinwald, 1988; Webster and Grab, 1988). The morphology of the endocytic organelles of *T. brucei* and *T. congolense* has been described in detail (Webster and

Grab, 1988; Webster, 1989; Webster and Fish, 1989). Serial sections through bloodstream form trypanosomes, which contained an endocytosed marker, have shown that the endocytic pathway appears to be a complex system of organelles with structural similarity to those found in mammalian cells (discussed by Webster, 1989b).

Based on the morphology of the organelles labelled with mAb 4E1 and the localization of these organelles to the posterior portion of the cell, the possible association of gp65 with the endocytic pathway was investigated. It had been well established that *T. brucei* and *T. congolense* were able to take up markers for endocytosis such as HRP and protein-colloidal gold conjugates, but no comparable studies had been carried out to indicate that *T. vivax* was capable of endocytosing these markers. Therefore, preliminary experiments were performed in order to assess: 1) whether *T. vivax* would remain viable under the incubation conditions; 2) if HRP and BSA-gold could be taken up by *T. vivax*; 3) how quickly HRP and BSA-gold were taken up; 4) whether different sized BSA-gold conjugates (5 and 10 nm conjugates) were taken up equally; and 5) whether, after uptake, these markers were sorted to different organelles due to differences in size.

Results presented in this chapter demonstrate that both BSA-gold and HRP are suitable markers to delineate the endocytic pathway in *T. vivax*. These markers were taken up rapidly, within 15 seconds, and cells remained viable for the duration of the experiment (up to 2 hours) as judged by motility. Both sizes of BSA-gold probe (5 nm and 10 nm gold conjugates) appeared to be taken up equally and could be found together in the same organelles at the various time points. This suggested that there was no sorting of the markers on the basis of particle size. Both HRP and BSA-gold could be seen associated with the outside surface of the cell and flagellum upon incubation in medium containing the endocytic markers. This phenomenon was also observed with *T. brucei* and *T. congolense*, even though, in these experiments, parasites were incubated in medium containing the markers at 4°C prior to warming to 37°C. The association of HRP with the surface of the cells was abundant and evenly distributed, which suggests that HRP had become bound to the surface. However, much less BSA-gold was cell surface-associated and it was also present in patches. In the previous studies, it was suggested that the association of BSA-gold with the surface of trypanosomes was an artifact of fixation since cells were fixed directly after incubation with BSA-gold without prior washing (Webster, 1989b). This explanation is valid, but as pointed out by Webster (1989a), although the uptake of BSA-gold is assumed to occur by a bulk-flow mechanism (i.e. no binding before uptake), it may not be a passive event. Since BSA appears to play a role in the uptake of fatty acids by bloodstream form trypanosomes (Voorheis, 1980), it may be taken into the cell by a specific process.

Initial endocytosis experiments with *T. vivax* were carried out as a time course in order to examine the progression of the marker through the cell and to establish suitable time points to be used for subsequent co-localization experiments with mAb 4E1. Results from these experiments show that BSA-gold was taken up rapidly from vesicles budding off the flagellar pocket membrane and that the marker progressed through the cell, from the flagellar pocket to the perinuclear region, where it became concentrated in what appeared to be roughly spherical organelles by 30 minutes. The morphology of the BSA-containing organelles could not be clearly distinguished. However, when HRP was used as a marker for endocytosis, the organelles could be seen more clearly (Fig. 4.3). A time course study to determine the uptake of HRP showed that what appeared to be the entire endocytic pathway could be labelled in 30 minutes. For this reason, only the 15 second and 30 minute time points are illustrated here. HRP, a soluble, fluid phase marker, was seen on the surface of the cells, flagellar pocket and inside small internal vesicles within 15 seconds of incubation. HRP was also observed in tubular structures which appear to be Golgi cisternae after a 30 minute incubation in the marker. The Golgi-association of HRP was also reported in *T. brucei* (Webster and Fish, 1989) and within one or more cisternae at the *trans* side of the Golgi after endocytosis of wheat germ agglutinin-HRP (WGA-HRP) by a rat pheochromocytoma cell line (Gonatas et al., 1984). Previous studies of endocytosis in *T. brucei* have shown that HRP occupies a larger internal cell volume (5% of the total cell volume) than BSA-gold (2% of the total cell volume) (Webster and Fish, 1989). In order to exclude the possibility that HRP uptake or the DAB reaction induced swelling in the endocytic organelles, thus increasing the apparent volume occupied by HRP, these investigators incubated *T. brucei* in the presence of both HRP and colloidal gold markers. Their results showed that the BSA-gold probe was excluded from some parts of the endocytic pathway, which may be a function of the larger size of the colloidal gold particles compared to the soluble HRP. Considering this information, it would have been desirable to use HRP as the endocytosed marker in co-localization studies, but when this was attempted, the DAB reaction appeared to abolish antigenicity since no labelling was achieved with mAb 4E1 (not shown).

Co-localization experiments were carried out using mAb 4E1 to label ultrathin sections of Lowicryl-embedded *T. vivax* which had been fixed at various times of incubation in medium containing BSA-gold. The results from these experiments show that the mAb 4E1 label and BSA-gold were co-localized to the greatest extent in cells which had been incubated with BSA-gold for 5 minutes. Co-localization of the two markers was reduced at time points after 5 minutes and none was noted at the 15 second time point. These results suggest that gp65 is located in an intermediate part of the endocytic pathway. The term "intermediate" is used to distinguish the early events of endocytosis involving the flagellar pocket and coated vesicles from later events involving lysosomes.

VSG, the major surface glycoprotein of trypanosomes, covers the entire surface of the cell, flagellar and flagellar pocket membrane. In *T. brucei*, VSGs have been shown to be anchored into the membrane via a GPI-linkage (discussed in section 1.5.4). A GPI-linkage has not been established for *T. vivax* VSGs, but cells biosynthetically labelled with ^{3}H-myristate incorporate label into VSG (Gardiner et al., 1987). Studies with *T. brucei* show that the VSG is constantly internalized from the surface, one estimate being 9.4% per hour (Coppens et al., 1987). A small proportion of the internalized VSG is degraded in lysosomes, some is lost from the surface by shedding and an estimated 95% is recycled back to the surface of the cell (Seyfang et al., 1990). On thin cryosections of *T. brucei*, using an anti-VSG antibody as a probe for internal VSG-containing structures, many tubules and vesicles in the posterior portion of the parasite have been shown to be labelled (Webster and Grab, 1988).

In an attempt to further characterize the gp65-containing organelles of *T. vivax*, a co-localization experiment was carried out using mAb 4E1 and a rabbit anti-VSG polyclonal antisera (RαVSG). Double antibody labelling was carried out on thin sections of *T. vivax* which had endocytosed BSA-gold for various times so that the antibody labels could be followed in relation to the progression of BSA-gold through the cell. In this experiment, more time points were included than in the previous experiment in order to cover the point of no co-localization of mAb 4E1 and BSA-gold (15 seconds) and the time of maximal co-localization (5 minutes) of the two markers. In doing this, it was hoped that the time point in which BSA-gold first encounters mAb 4E1-labelled organelles could be determined.

The results from the double antibody labelling experiment show that BSA-gold and VSG entered the cell via the flagellar pocket within 1 minute. Although not clearly demonstrated in Fig. 4.5, BSA-gold and VSG are able to enter the cell in the same vesicles, some of which resemble the clathrin-coated vesicles of mammalian cells. This has also been demonstrated in *T. brucei* (Webster, 1989; Seyfang et al., 1990). Many internal structures were labelled with the RαVSG probe, including tubules, vesicles and cisternae of the Golgi apparatus. Presumably, the VSG-containing organelles are representative of both the endocytic/recycling pathway and the biosynthetic/secretory pathway, as there is no way to distinguish incoming VSG from that destined for export in this experiment. Nonetheless, none of these RαVSG-labelled structures contained mAb 4E1 label. The mAb 4E1-labelled organelles appeared to be located closer to the nucleus than the VSG-containing structures. Therefore, as a general statement, gp65 does not appear to play a role in VSG recycling, or transport of newly synthesized molecules to the surface and thus may only be present in organelles that are involved with the processing of endocytosed material destined for degradation.

By examining the relative positions of the three markers, BSA-gold, RαVSG and mAb 4E1 label in *T. vivax* with increasing times of incubation in the endocytic marker, several observations could be made. First, although BSA-gold could be taken up by the cell initially in the vesicles which contained internalized VSG, the RαVSG and BSA-gold markers were not co-localized after 2 minutes. Therefore, it would appear that a sorting event had occurred within 2 minutes after endocytosis, at which point, material destined for the late endosomal pathway is separated from VSG. Presumably, most of the VSG returns to the surface as predicted by kinetic data (Seyfang et al., 1990), but it is possible that VSG which is to be degraded goes to lysosomes by a different pathway. Apart from the initial co-localization of RαVSG and BSA-gold, the endocytosed marker was found in distinct organelles until it co-localized with the mAb 4E1 label by 4 minutes as described above. Thus, BSA-gold appeared to enter the gp65-containing structures between 3 and 4 minutes after the start of incubation in this label.

In trypanosomes, the components of the endocytic pathway have not been defined, due to the lack of immunochemical and cytochemical markers. Several proteins have been identified, to which there are specific antibodies, that should aid in the dissection of the pathway. However, the species-specificity of the lysosomal marker, a 33 kDa cysteine protease of *T. congolense* (Mbawa et al., 1991), and the *T. vivax*-specificity of mAb 4E1 prevent the simultaneous definition of the compartments they mark in one species of trypanosome. Therefore, the only comparisons that can be made are with mammalian cells, where the endocytic pathway has been fairly well characterized.

The endocytic components of mammalian cells can be sub-divided into the pre-endosomal compartment consisting of coated and non-coated vesicles, early endosomes, late endosomes, and lysosomes. Endosomes have been fairly well characterized in terms of their function but they are heterogenous in terms of morphology and cellular distribution. As a general description they are tubulo-vesicular structures, with an average size of 0.3 µm but can be as large as 1 µm (Helenius et al., 1983). The acidic nature of endosomes has been established using the pH sensitivity of fluorescein-labelled ligands (Tycko and Maxfield, 1982; Murphy et al., 1984; van Renswoude et al., 1982) and using the pH-dependent fusion of Semliki Forest Virus (SFV) (Marsh et al., 1983) which occurs at below pH 6.0. These studies with mammalian cells showed that endocytosed material entered an acidic compartment within 5 minutes after internalization. In addition to studies *in vivo*, the acidity of endosomes has also been established by *in vitro* methods

(Galloway et al., 1983). The acidic environment of the endosome is maintained by an ATP-dependent proton pump (Galloway et al., 1983; Merion et al., 1983; Yamashiro et al., 1984; Mellman et al., 1986). Two functionally distinct populations of endosomes, termed early and late endosomes, have been isolated and characterized (Schmid et al., 1988). Early endosomes, which can be labelled following a short exposure to an endocytosed marker, typically 5 minutes, are the site at which many receptor-ligand complexes dissociate. The acidic environment is instrumental in the dissociation of receptors from ligands, and free receptors are recycled back to the surface (Helenius et al., 1983; Gruenberg et al., 1989; Griffiths et al., 1989). Late endosomes are labelled with longer exposures to markers, 10 to 15 minutes, and are involved in delivering molecules to the lysosomes (Schmid et al., 1988). Ligands enter cells and pass from early endosomes, where sorting occurs, to late endosomes if material is destined for degradation. Some receptors, such as the transferrin receptor are recycled to the surface in less than 3 minutes (Klausner et al., 1983; Ciechanover et al., 1983; Dautry-Varsat et al., 1983; Townsend et al., 1984). Since the recycling has been shown to occur from the early endosome, this supports the idea that early endosomes are formed very quickly after internalization of endocytosed material. Late endosomes could be labelled with HRP after a 15 minute incubation, but using ^{35}S-SFV to load late endosomes, it was found that 10% of the virus had entered the lysosomes while 75% of the label was still associated with the late endosomes at 15 minutes (Schmid et al., 1988). By examining the protein profiles of early and late endosomes many common proteins can be seen, but distinct proteins can be identified as well (Schmid et al., 1988). Interestingly, these protein profiles also differ from that of the plasma membrane, which suggests that early endosomes are not simply derived from the budding off of the surface membrane but require input of different proteins (Schmid et al, 1988).

Lysosomes are acidic organelles rich in hydrolases, where most biological macromolecules can be degraded (for a review, see Kornfeld and Mellman, 1989). They can be viewed as the product of two converging pathways: the endocytic pathway and the biosynthetic/secretory pathway. Whether lysosome biogenesis occurs by the maturation model (reviewed by Murphy, 1991) or the vesicle transport model (reviewed by Griffiths and Gruenberg, 1991), a fusion event must occur whereby newly synthesized lysosomal hydrolases from the Golgi apparatus are delivered to the immature lysosome (Kornfeld and Mellman, 1989; Stoorvogel et al., 1989). The point along the endocytic pathway to which these enzymes are delivered is still controversial.

The division of the endocytic pathway into component parts is an oversimplification which alludes to the concept of stable organelles with defined characteristics. For example, the ability to segregate an early endosomal population from late endosomes and lysosomes by free-flow electrophoresis and biochemical characteristics (Schmid et al., 1988), suggests that these organelles are distinct entities It also implies that vesicular traffic must occur between the organelles in order to lead to the delivery of endocytic material to the lysosomes. The maturation model maintains that by various fusion and fission events with gradual acidification of the endocytic organelle, endocytosed material will eventually be in an acidic, hydrolase rich environment. Rate-limiting steps at certain points in the pathway could lead to the apparent pauses that would allow the isolation of relatively distinct organelle populations (Murphy, 1991).

Many unresolved questions remain in our understanding of the mechanism of the endocytic pathway of mammalian cells. It is likely that there are different types of endocytic mechanisms which may accommodate both the maturation model and the vesicle transport model. Even more likely is that individual cell types have variations of the mechanisms described. In trying to define the *T. vivax* gp65-containing organelles based on criteria established for mammalian cells, it would appear that ese organelles are similar to late endosomes. No information has been obtained about the degree of acidification of the mAb 4E1-labelled organelles. However, BSA-gold and mAb 4E1 were co-localized within 4-5 minutes, an event that occurred after the apparent sorting of VSG. If the assumption is made that trypanosomes possess a compartment comparable to early endosomes, where molecules destined for recycling are sorted from those destined for degradation, then BSA-gold would have been in this compartment within 1-2 minutes of internalization. where it could be co-localized with VSG. Therefore, by the criteria established for mammalian cells, the gp65-associated organelles would be more similar to late endosomes than early endosomes In a maturation type model, gp65 would be delivered to this compartment (perhaps newly synthesized from the trans-Golgi network or recycled from lysosomes) by a fusion event, where it would remain for approximately 15 minutes. As gp65 does not appear to be located in lysosomes, it may be removed from maturing vesicles by a fission event and recycled back to join an earlier endosomal compartment. Alternatively, the possibility exists that lysosome-associated gp65 is degraded and no longer recognized by the monoclonal antibody. There is no evidence that gp65 is Golgi-associated and thus does not appear to be present in vesicles which have budded from the Golgi apparatus which carry newly synthesized proteins. However, gp65 may have a role in the delivery of molecules to the endocytic pathway, or perhaps it is needed as a recognition molecule for the targeting of subsequent molecules or vesicles.

CHAPTER 5

Further biochemical characterization of gp65

5.1 Introduction

Results presented in Chapter 4 clearly demonstrate an association of gp65 with an endocytic compartment of *T. vivax*. One of the original aims of this study was to identify an invariant antigen that may be accessible to antibody binding. In order for gp65 to be accessible to external reagents, at least part of it must be lumenally oriented within these endocytic vesicles. In this chapter, the biochemical characteristics of gp65 are investigated further by attempting to determine the orientation of gp65 on the membrane, to further assess the relationship of gp65 and p22, and finally to partially purify gp65 so that a limited N-terminal sequence may be obtained. The data obtained from these experiments are discussed in the context of possible function(s) of the gp65 molecule.

5.2 Materials and Methods

5.2.1 Protease Protection Experiments.

Freshly prepared membranes (see 3.3.10) were gently suspended in TBS (10 mM Tris-HCl [pH 8.0], 150 mM NaCl) using a loose fitting Dounce homogenizer. 500 µl aliquots of membrane suspension (250 µg protein) were incubated at 37°C for 1 hour with either trypsin (TPCK-treated, Sigma) (100 µg/ml), endoproteinase Asp-N

(Boehringer Mannheim, Germany) (1.6 μg/ml), or endoproteinase Glu-C (Boehringer Mannheim, Germany) (40 μg/ml) in the presence or absence of 0.1% (v/v) Triton X-100. The reactions were stopped by adding 500 μl double-strength Laemmli sample buffer and boiling for 5 minutes. 40 μl aliquots were used for SDS-PAGE and antigen detection by immunoblotting.

5.2.2 Cross-linking of gp65 in a membrane fraction.

50 μl of membrane suspension, prepared as in 5.2.1, was added to 450 μl of 50 mM triethanolamine (pH 8.2), 100 mM NaCl, 1 mM EDTA, 5% (w/v) sucrose and 5 μg of each of the protease inhibitors, antipain, leupeptin and E-64. The membrane permeable cross-linker, dithio*bis*(succinimidyl propionate), DSP, (50 mM in DMSO), was added to the diluted membrane suspension to give a final concentration of 0.5 mM DSP and 1% (v/v) DMSO (Baskin and Yang, 1982). For treatment with 3,3'-dithio*bis*(sulfo-succinimidyl propionate), DTSSP, the membrane impermeable cross-linker, 5 μl of a 100 mM stock in distilled H_2O was added to 500 μl diluted membrane suspension to give a final DTSSP concentration of 1 mM. Membranes were incubated at room temperature for 2 hours in the presence and absence of cross-linker, then pelleted at 100,000*g* for 45 minutes in a 70.1 Ti rotor (Beckman), and boiled for 3 minutes in Laemmli sample buffer with, or without, 50 mM DTT. Samples were run on SDS-PAGE under reducing and non-reducing conditions, blotted and probed with mAb 4E1.

5.2.3 Treatment of membranes with Na_2CO_3 and DTT.

Freshly prepared membranes were treated with 0.2 M Na_2CO_3, (pH 11.5) according to the method of Fujiki et al., (1982) as described in Chapter 3, with the addition of 50 mM DTT. Pellet and supernatant fractions were analyzed for gp65 and p22 by immunoblotting with mAb 4E1.

5.2.4 Isoelectric focusing two-dimensional gels.

Trypanosome membrane proteins, which had been bound to and eluted from Con A-Sepharose by specific elution with mannose, were used for two-dimensional (2-D) isoelectric focusing gels since this sample was enriched for gp65 and p22 125 µg total protein was precipitated by the chloroform/ methanol/water method (Wessel and Flügge, 1984). The protein precipitate was dissolved in 35 µl of sample buffer: 9 M urea, 5% (v/v) 2-mercaptoethanol, 4% (v/v) Nonidet P-40, 2% (v/v) pH 9-11 ampholines (Pharmacia, Uppsala, Sweden) and 30 µl was applied to the first dimension, isoelectric focusing tube gel of the two-dimensional ISO-DALT electrophoretic separation system of Anderson and Anderson (1978a and b) and run for 10 000 volt/hours. Ampholines in the first dimension tube gel were a mixture of 70% (v/v) pH 3.5-10 and 30% (v/v) pH 4-6 (Pharmacia). Tube gels were equilibrated in Laemmli sample buffer for 5 minutes and placed on the top of a 5-15% gradient gel and electrophoresed in the second dimension at 20 mA/gel for 6 hours. Some of the gels were stained using Coomassie blue to visualize the proteins, others were immunoblotted and developed using mAb 4E1.

5.2.5 Cleveland digests of gp65 and p22.

Two hundred micrograms of protein eluted from Con A-Sepharose were electrophoresed under reducing conditions on preparative 10-15% SDS-PAGE minigels (1.0 mm). These gels were stained with Coomassie blue, and destained until the background was light blue. Bands in the region of gp65 and p22 were excised. These gel slices were cut to a width suitable to fit into the wells of the second gel and equilibrated for 5 minutes in the equilibration/protease dilution solution described by Cleveland (1983), then placed into wells of a 1.5 mm SDS-PAGE gel (14 ml, 20% separating gel, 5% stacking gel). For electrophoresis, the anode buffer was 200 mM Tris-HCl (pH 8.0), 0.1% (w/v) SDS and the cathode buffer was 100 mM Tris, 100 mM Tricine and 0.1% (w/v) SDS after the procedure of Schägger and von Jagow (1987). Gel slices were overlaid with 10 µl of gel slice overlay buffer (Cleveland, 1983). *Staphylococcus aureus* V8 protease (SV8) or trypsin were diluted in equilibration buffer and 20 µl of the appropriate dilution was added to wells and electrophoresis was carried out at 50 volts (constant voltage) until proteins entered 1 cm into the stacking gel. Electrophoresis was stopped for 30 minutes to allow digestion to take place, then resumed at 50 volts until the separating gel was reached and the voltage was increased to 150 V until completion. Gels were blotted and developed with mAb 4E1 to detect gp65, p22 and immunoreactive protease digestion products as described above (section 3.2.5).

5.3.3 Cleveland Mapping

To try to identify a structural relationship between gp65 and p22, gel slices containing either protein were subjected to proteolytic digestion with endo Glu-C (SV8) and trypsin at various concentrations (Fig. 5.4, A and B). Only a small proportion of gp65 was digested with 0.05 µg of SV8 (Fig. 5.4 A, lane 4), but when 0.3 µg, 1.0 µg and 3.0 µg were used (Fig. 5.4 A, lanes 5, 6, and 7, respectively), increasing amounts of gp65 were digested in the time allowed for the experiment. The only detectable digestion product had a molecular mass of approximately 20-21.5 kDa. By comparing the intensity of the band of the SV8 digestion product when 0.3 µg and 1.0 µg SV8 were used (Fig. 5.4 A, lanes 5 and 6), with the intensity of this band when 3.0 µg SV8 was used (Fig. 5.4 A, lane 7), there appears to be less of the digestion product after treatment of gp65 with 3.0 µg SV8. This suggests that there are other SV8 cleavage sites present in the 20 kDa digestion product but that resulting peptides were not detectable by immunoblotting. No detectable breakdown products were obtained after treatment of p22 with any concentration of SV8 (Fig. 5.4 A, lanes 8-11). However, there appears to be slightly less p22 present in the lane which received 3.0 µg SV8, indicating that p22 may have a cleavage site, although possible cleavage products of p22 were not detected by immunoblotting.

Trypsin was less efficient in digesting gp65 since much higher amounts of enzyme were needed for digestion (0.5 - 10 µg), and complete digestion of the gp65 was not achieved (Fig. 5.4 B, lanes 4-7). The only detectable trypsin cleavage product of gp65 was approximately 17 kDa and was only present when volume of packed RCA-agarose beads [pre-equilibrated with 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% (v/v) Triton X-100]. Unbound proteins were removed by washing with 50 volumes of buffer and bound proteins were eluted with 0.5 M galactose in 10 mM Tris-HCl (pH 7.4), 150 mM NaCl with 0.01% (v/v) Triton X-100. The concentration of protein in the pooled DE-52 fractions or the RCA-agarose fractions was not determined. Instead, 5 μl of each sample was used for electrophoresis, 1/10 the volume of the fractions electrophoresed in Fig. 5.7 B.

5.3 Results

5.3.1 Membrane orientation of gp65

To investigate the orientation of gp65 in the membrane, the accessibility of the molecule to exogenous proteases as well as to membrane permeable and impermeable cross-linking reagents was determined (Figs. 5.1 and 5.2, respectively). For the protease protection experiment, aliquots of a membrane fraction were treated with various proteases in the presence and absence of Triton X-100, and the fate of gp65 was then detected by immunoblotting. Treatment of membranes with trypsin had no effect on gp65 unless Triton X-100 was present in the incubation mixture (Fig. 5.1, lane 4). This suggests that the cleavage site(s) for trypsin is on the lumenal face of the vesicles. However, gp65 is partially cleaved by both endo Asp-N and endo Glu-C in the absence of detergent and is fully cleaved by both enzymes when Triton X-100 is present (Fig. 5.1, lanes 6 and 7, respectively). This suggests that there are cleavage sites for these two enzymes on both sides of the vesicles.

Figure 5.1

Protease protection of gp65.

Membranes were treated with trypsin, endoproteinase Asp-N or endoproteinase Glu-C in the presence or absence of 0.1% Triton X-100. Samples were run on SDS-PAGE, blotted and probed with mAb 4E1. Lane 1, membranes (M) treated with 0.1% Triton X-100; lanes 2, 5 & 8 are mock treated controls for endogenous protease activity; lanes 3, 6 & 9 contain membranes treated with trypsin (100 μg/ml), endoproteinase Asp-N (1.6 μg/ml), and endoproteinase Glu-C (40 μg/ml), respectively; lanes 4, 7 & 10 are trypsin, endo Asp-N and endo Glu-C treated membranes, respectively, with the addition of 0.1% Triton X-100. 10 μg of total membrane protein was loaded in each lane.

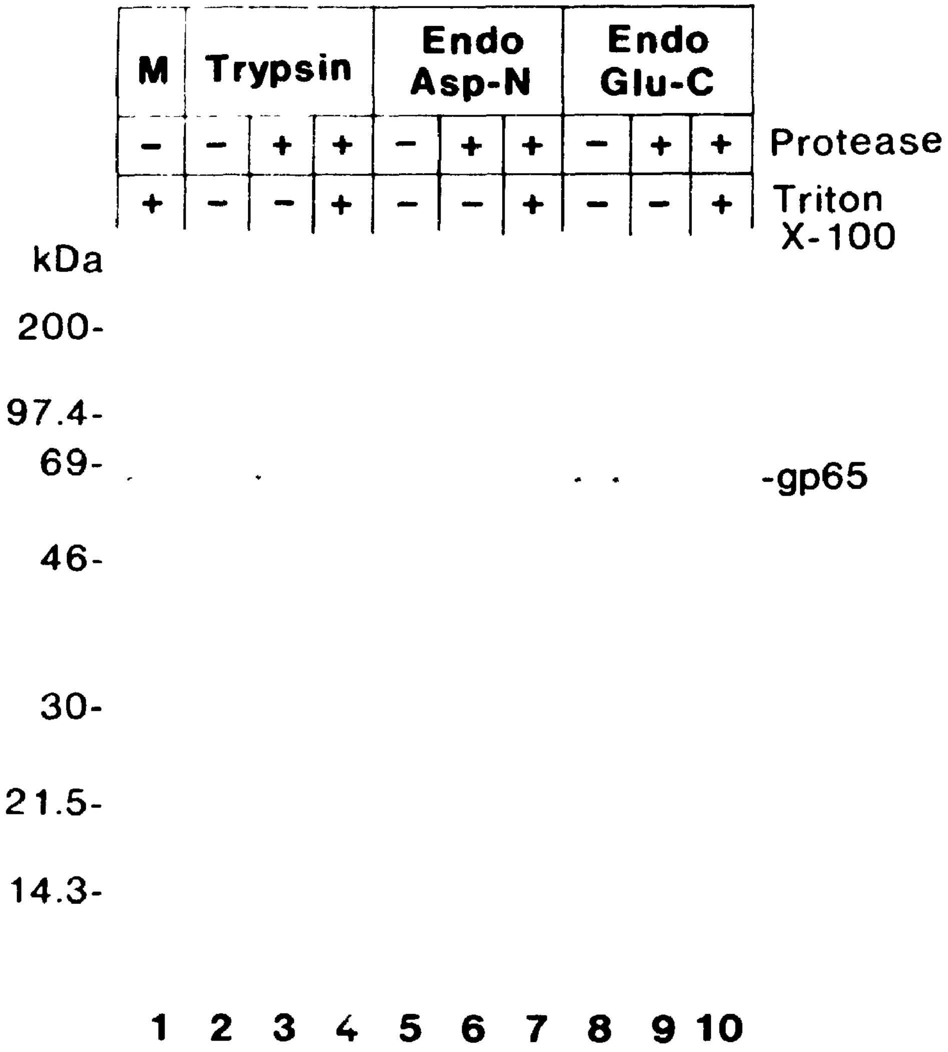
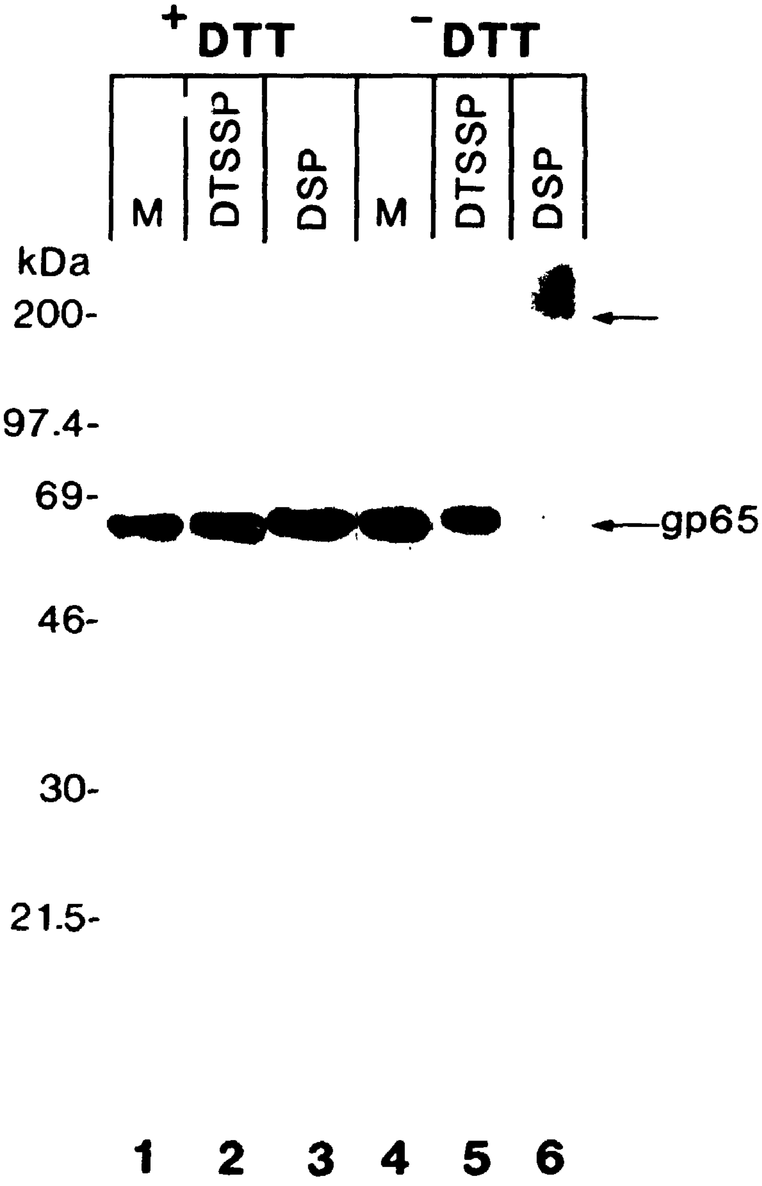

M
Trypsin
Endo Asp-N
Endo Glu-C
– – + + – + + – + + Protease
+ – – + – – + – – + Triton X-100
kDa
200-
97.4-
69-
46-
30-
21.5-
14.3-
-gp65
1 2 3 4 5 6 7 8 9 10

Figure 5.2

Chemical cross-linking of gp65 in membranes.

Membranes (250 µg protein) were either mock-treated (lanes 1 & 4), treated with the membrane impermeable cross-linker, DTSSP (lanes 2 & 5), or the membrane permeable cross-linker, DSP (lanes 3 & 6). 30 µg total membrane protein was electrophoresed under reducing conditions (+DTT, lanes 1-3), or non-reducing conditions (-DTT, lanes 4-6), blotted and probed with mAb 4E1. In lane 6, the upper arrow indicates the high molecular weight species obtained using the membrane permeable cross-linker DSP.


+DTT
-DTT
M
DTSSP
DSP
M
DTSSP
DSP
kDa
200-
97.4-
69-
46-
30-
21.5-
gp65
1
2
3
4
5
6

Membranes treated with Triton X-100 (Fig. 5.1, lanes 1) or incubation of membranes in the absence of proteolytic enzymes (Fig. 5.1, lanes 2, 5, and 8) demonstrates that gp65 is not degraded under control conditions.

Cross-linking studies using membrane permeable (DSP), and impermeable (DTSSP) cross-linkers demonstrate that only DSP cross-links gp65 efficiently (Fig. 5.2, lane 6). When cross-linked membranes were electrophoresed under reducing conditions (Fig. 5.2, lanes 2 and 3), as a control for the reversibility of the cross-linking, gp65 was detected after treatment with both DSP and DTSSP. Untreated membranes were also run under reducing and non-reducing conditions (Fig. 5.2, lanes 1 and 4) to ensure that the gp65 was not cross-linked before treatment. These results show that gp65 is inaccessible to cross-linking unless the cross-linker can traverse the membrane.

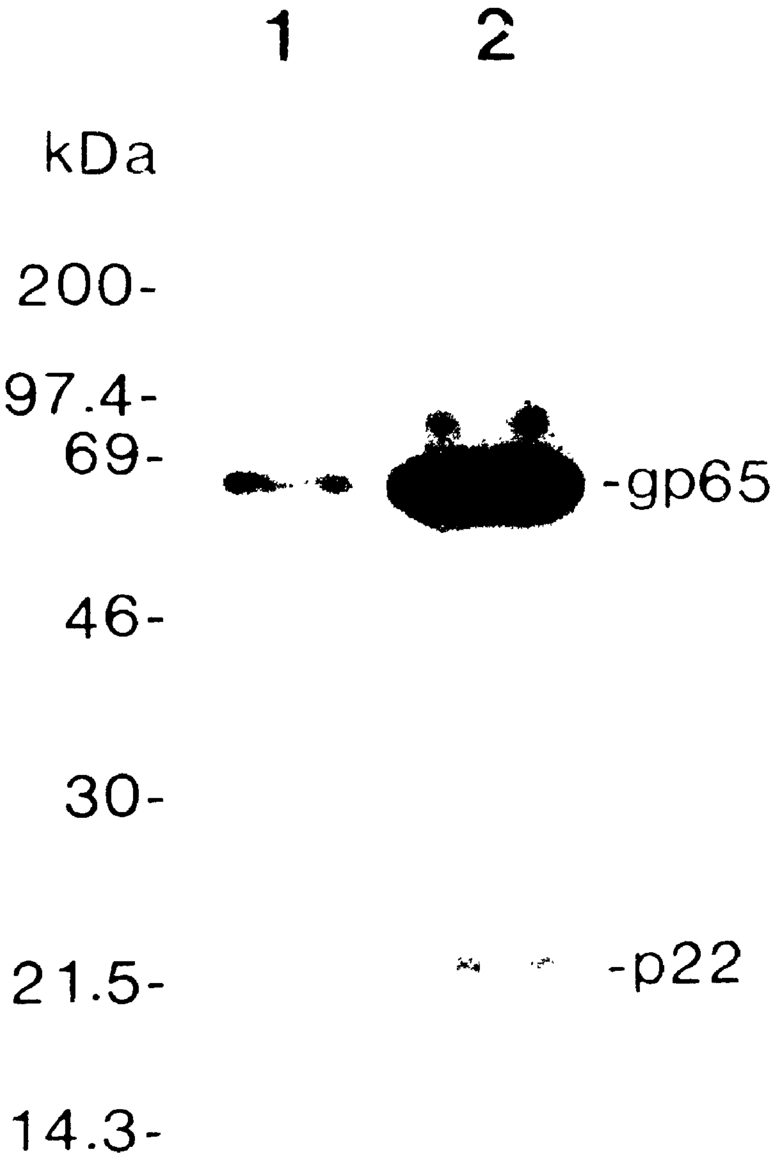
5.3.2 Both gp65 and p22 are tightly associated with the membrane.

Membranes were treated with 0.2 M Na_2CO_3 containing 50 mM dithiothreitol (DTT) at alkaline pH to determine if this would effect the solubility of either gp65 or p22 since they are associated by disulfide linkage. The results show that the addition of a high concentration of DTT to Na_2CO_3 did not aid in the release of either the entire gp65 or the p22 portion from membranes (Fig. 5.3).

Figure 5.3

Both gp65 and p22 are tightly associated with the membrane.

Membranes were extracted with 0.2 M Na_2CO_3 (pH 11.5) containing 50 mM DTT, for 1 hour on ice. Membranes were separated from solubilized proteins by centrifugation at 120,000 g for 1 hour. Aliquots of the resulting supernatant (lane 1) and pellet (lane 2) fractions were separated on a 10-15% SDS-PAGE gel and gp65 and p22 were detected by immunoblotting with mAb 4E1. 25 μg total protein was loaded in each lane.


1
2
kDa
200-
97.4-
69-
46-
30-
21.5-
14.3-
-gp65
-p22

5.3.3 Cleveland Mapping

To try to identify a structural relationship between gp65 and p22, gel slices containing either protein were subjected to proteolytic digestion with endo Glu-C (SV8) and trypsin at various concentrations (Fig. 5.4, A and B). Only a small proportion of gp65 was digested with 0.05 µg of SV8 (Fig. 5.4 A, lane 4), but when 0.3 µg, 1.0 µg and 3.0 µg were used (Fig. 5.4 A, lanes 5, 6, and 7, respectively), increasing amounts of gp65 were digested in the time allowed for the experiment. The only detectable digestion product had a molecular mass of approximately 20-21.5 kDa. By comparing the intensi.¸ f the band of the SV8 digestion product when 0.3 µg and 1.0 µg SV8 were used (Fig. 5.4 A, lanes 5 and 6), with the intensity of this band when 3.0 µg SV8 was used (Fig. 5.4 A, lane 7), there appears to be less of the digestion product after treatment of gp65 with 3.0 µg SV8. This suggests that there are other SV8 cleavage sites present in the 20 kDa digestion product but that resulting peptides were not detectable by immunoblotting. No detectable breakdown products were obtained after treatment of p22 with any concentration of SV8 (Fig. 5.4 A, lanes 8-11). However, there appears to be slightly less p22 present in the lane which received 3.0 µg SV8, indicating that p22 may have a cleavage site, although possible cleavage products of p22 were not detected by immunoblotting.

Trypsin was less efficient in digesting gp65 since much higher amounts of enzyme were needed for digestion (0.5 - 10 µg), and complete digestion of the gp65 was not achieved (Fig. 5.4 B, lanes 4-7). The only detectable trypsin cleavage product of gp65 was approximately 17 kDa and was only present when

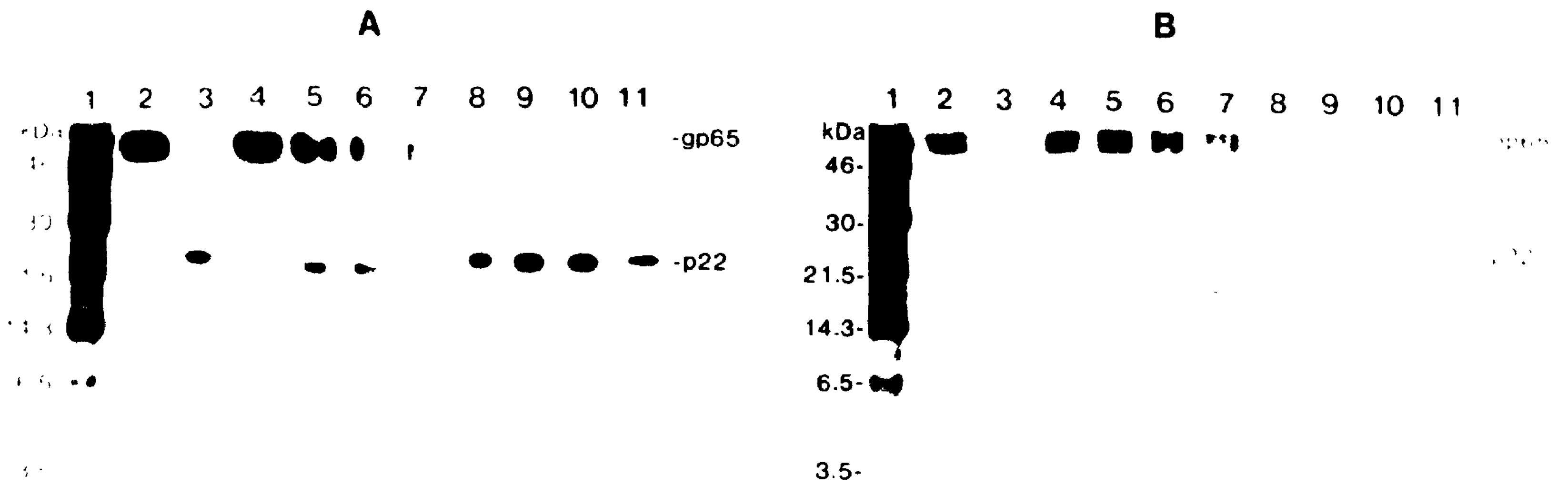
Figure 5.4

Cleveland digests of gp65 and p22.

Gel slices containing either gp65 and p22 were treated with varying amounts of endoproteinase Glu-C (SV8) (A) or trypsin (B), and resulting cleavage products were separated on a 20% SDS-PAGE gel, electroblotted and developed with mAb 4E1.

(A) Undigested gel slices containing gp65 (lane 2) and p22 (lane 3). Lanes 4-7, gp65 treated with 0.05 µg, 0.3 µg, 1.0 µg and 3.0 µg SV8 respectively; lanes 8-11 contain gel slices of p22 with the same amount of SV8 added (0.05 µg, 0.3 µg, 1 0 µg and 3.0 µg) respectively. Lane 1 contains ^{14}C-methylated rainbow low molecular weight protein markers (10 µl).

(B) Undigested gel slices containing gp65 (lane 2) and p22 (lane 3). Lanes 4-7, gp65 treated with 0.5 µg, 1.0 µg, 5 µg, and 10 µg trypsin respectively; lanes 8-11 contain gel slices of p22 with the same amounts of trypsin (0.5 µg, 1.0 µg, 3.0 µg, and 10 µg) respectively. Lane 1 contains ^{14}C-methylated low molecular weight protein markers.


A
B
1
2
3
4
5
6
7
8
9
10
11
-gp65
-p22
kDa
46-
30-
21.5-
14.3-
6.5-
3.5-

SV8

Trypsin

5 µg or 10 µg of trypsin was used (Fig. 5.4 B, lanes 6 and 7). Trypsin did not appear to digest the p22 molecule (Fig. 5.4 B, lanes 8-11).

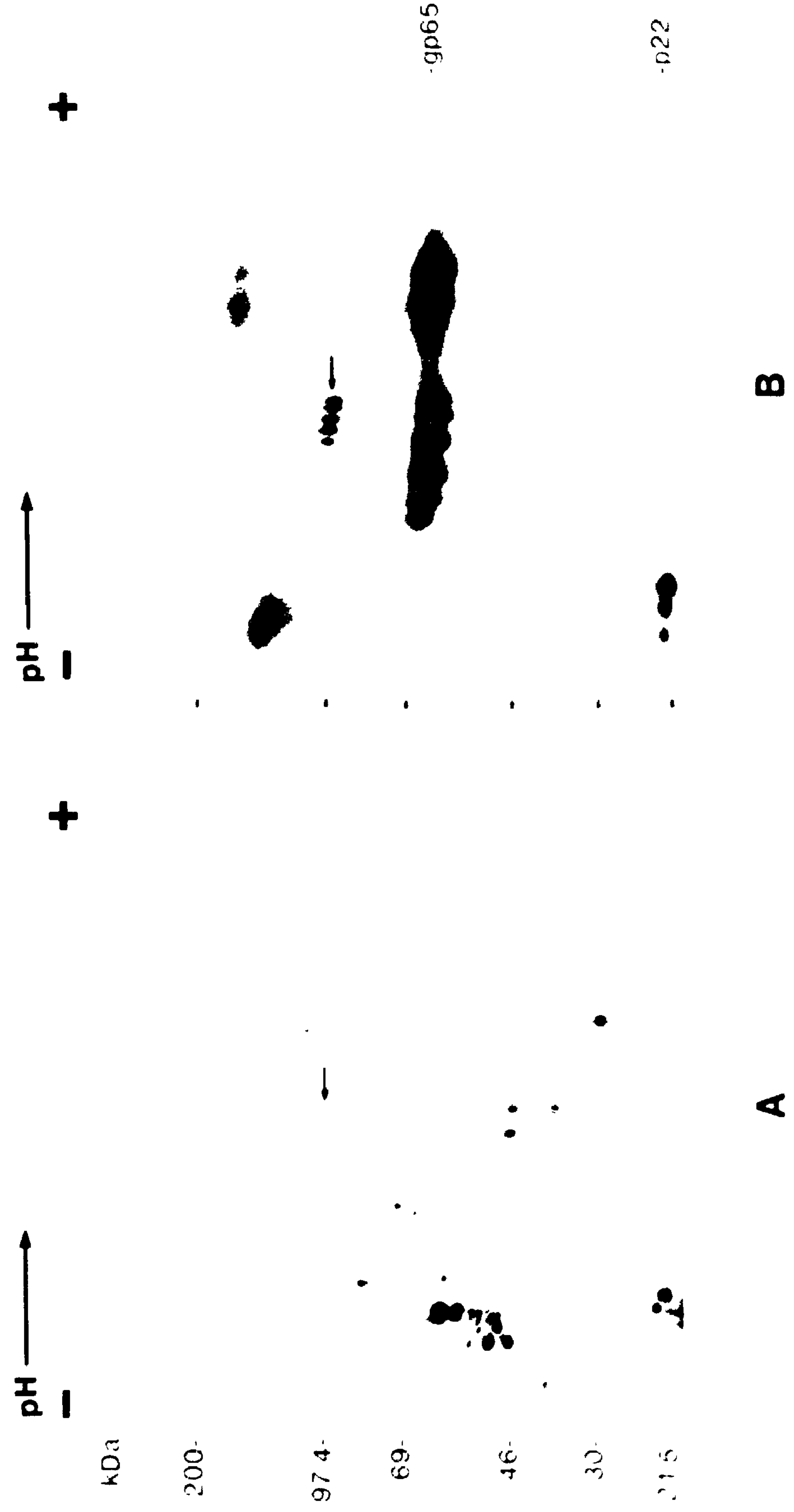
5.3.4 IEF and Two-dimensional Gels.

Proteins eluted from Con A-Sepharose were separated by isoelectric focusing and SDS-PAGE in order to try to resolve gp65 from other proteins of similar molecular mass and to get an indication of its isoelectric point (pI). Five times more protein was used for the two-dimensional (2-D) gels (125 µg) than was routinely used for separation on one-dimensional gels (25 µg), and samples were run in duplicate One of the gels was stained for protein by Coomassie blue (Fig. 5.5 A), and the other was immunoblotted and gp65 detected with mAb 4E1 (Fig. 5.5 B). The position of gp65 on the Coomassie blue stained gel could not be determined by comparing it to the immunoblot. This was anticipated since gp65 does not stain well on one-dimensional gels. The resolution pattern of the immunoreactive proteins was quite complex. The gp65 appeared to resolve to two distinct species as detected on the immunoblot: one that consisted of 5-6 spots ranging from the approximately neutral to the acidic (-) of the gel, and the second species that was resolved more to the basic end (+) of the gel. In addition, the more basic species appeared to run slightly slower on the second dimension gel. The p22 breakdown product could be detected at the acidic end of the gel. In addition to gp65 and p22, another immunoreactive species of approximately 100 kDa, which resolved to 5 spots directly above the neutral gp65

Figure 5.5

Two-Dimensional IEF gels of proteins eluted from Con A-Sepharose.

Duplicate samples of 125 μg of proteins eluted from Con A-Sepharose were separated in an isoelectric focusing tube gel in the first dimension, followed by a 5-15% gradient SDS-PAGE gel in the second dimension. One gel was stained by Coomassie blue (A) and the other was blotted and developed with mAb 4E1 (B). The position of gp65 and p22 are marked on the immunoblot. The position of a higher molecular weight immunoreactive protein is indicated by an arrow on both A and B.


kDa
200-
97.4-
69-
46-
30-
21.5
pH
−
+
A
B
gp65
p22

spots, was observed (Fig. 5.5 B, indicated with an arrow). The similarity in resolution patterns of the 100 kDa protein to gp65 suggests that it may be a related molecule.

5.3.5 DE-52 Chromatography of membrane proteins eluted from Con A-Sepharose.

Figure 5.6 shows the profile of the Con A-binding proteins eluted from DE-52 with a NaCl gradient. The peak of protein eluted with salt concentrations of between 180-280 mM NaCl. Aliquots from selected fractions were separated under reducing conditions and either stained for protein with Coomassie blue (Fig. 5.7 A) or blotted and probed with mAb 4E1 for the detection of gp65 (Fig. 5.7 B). The blot (Fig. 5.7 B) showed that gp65 was eluted in fractions 11-15, corresponding to a salt concentration between the range of 118-165 mM NaCl. Therefore, gp65 was enriched in these fractions as it eluted slightly before the main protein peak.

Fractions 10-15 were pooled and gp65 was enriched further by binding to RCA agarose (Fig. 5.8). The protein profiles of the various fractions shown in Fig. 5.8 A do not appear to be very different, however gp65 was highly enriched in the fraction that was bound and eluted from RCA-agarose, as judged by the immunoblot (Fig. 5.8 B). The band in the silver stained gel, indicated with an arrow (Fig 5.8 A), may correspond to gp65.

Figure 5.6

Fractions obtained from a DE-52 Cellulose Chromatography Column

Membrane proteins eluted from Concanavalin A-Sepharose with α-methyl mannoside were separated on a DE-52 cellulose column by binding to the matrix and eluting with a 20 - 400 mM NaCl gradient. 500 μl fractions were collected and the relative protein content in each fraction was estimated by determining the absorbance at 280 nm.

DE-52 Chromatography

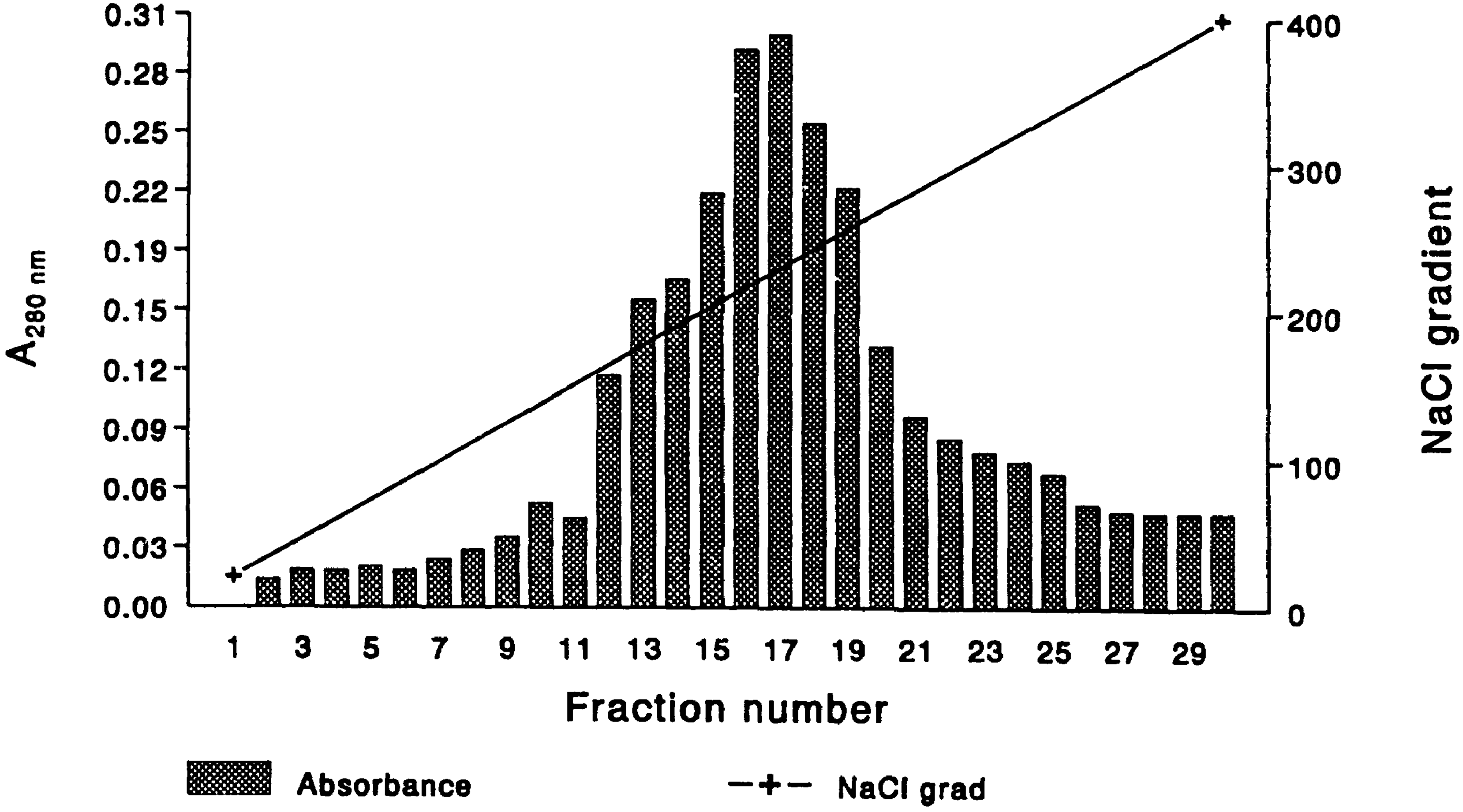


Figure 5.7

Enrichment for gp65 by DE-52 Chromatography

50 µl aliquots of selected fractions from the DE-52 column were electrophoresed on duplicate SDS-PAGE gels. One of the gels was stained with Coomassie blue (A) and the other was electroblotted and probed with mAb 4E1 (B). The number of the fraction loaded in each lane is indicated in the figure. 25 µg of Con A proteins, the column starting material, were electrophoresed in the first lane (sm). The position of the protein molecular weight standards (Amersham) is indicated in both A and B.

DE-52 Chromatography

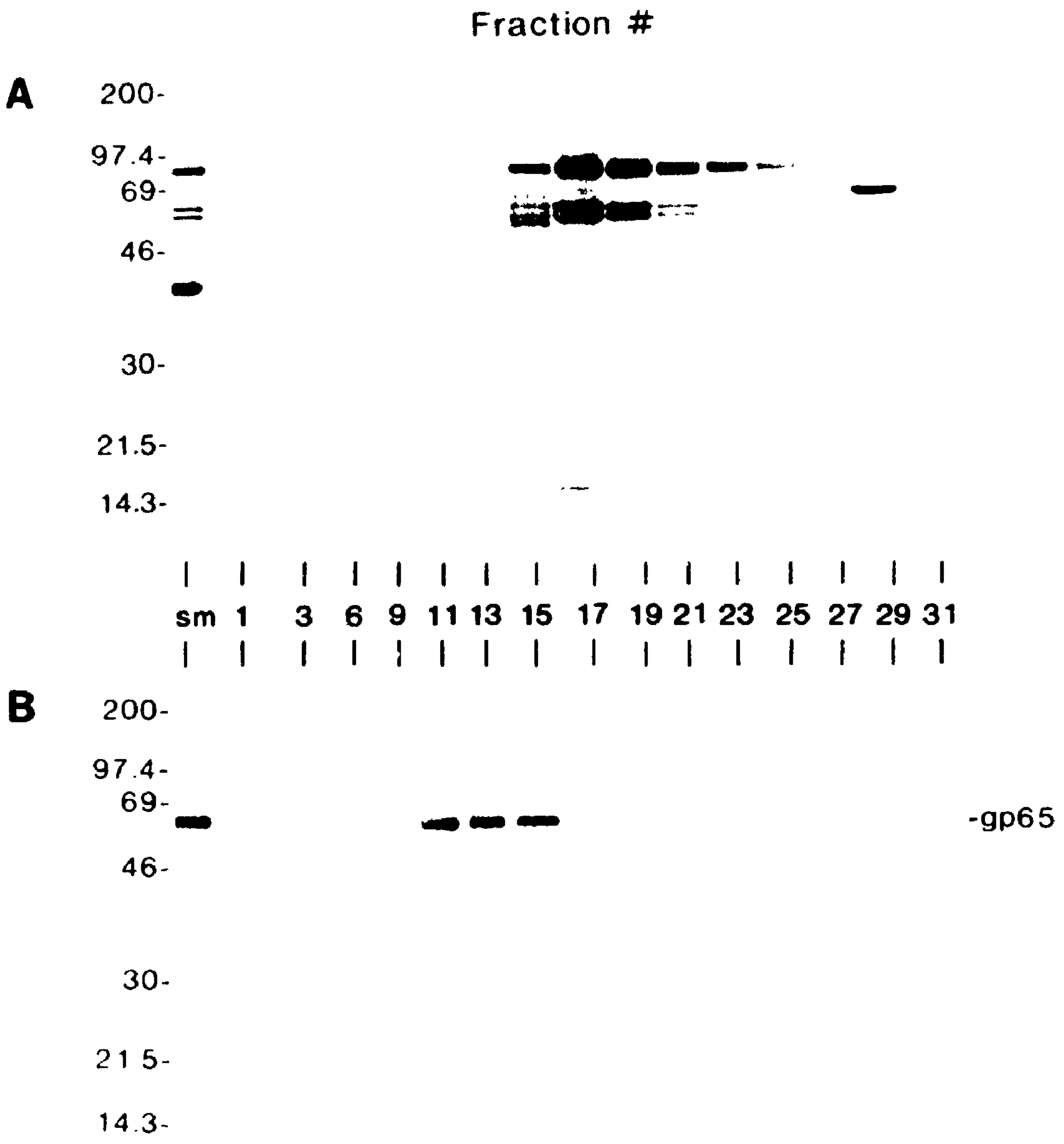
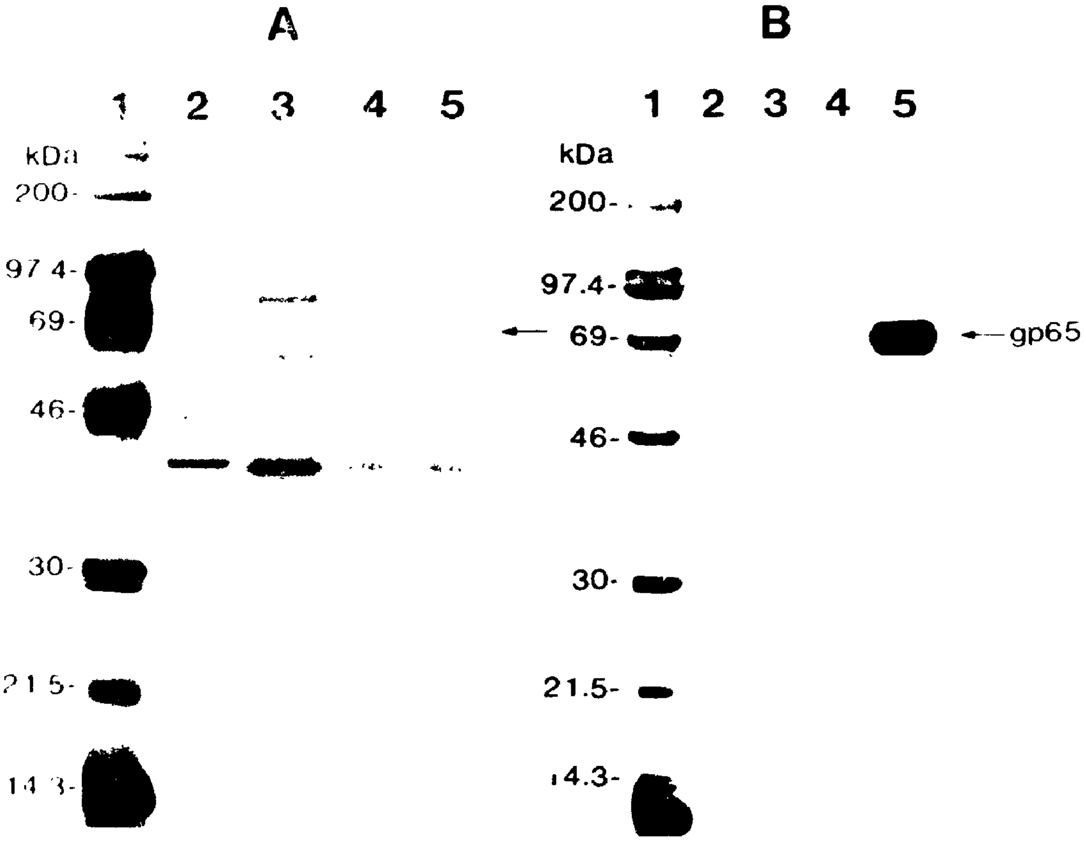

Fraction #
A
200-
97.4-
69-
46-
30-
21.5-
14.3-
sm
1
3
6
9
11
13
15
17
19
21
23
25
27
29
31
B
200-
97.4-
69-
46-
30-
21 5-
14.3-
-gp65

Figure 5.8

Further enrichment of gp65 by binding to RCA-agarose

Pooled gp65-containing fractions (# 10-15) from the DE-52 column were bound to RCA-agarose. Unbound proteins were removed by extensive washing and bound proteins were eluted with 0.5 M galactose. Resultant fractions were electrophoresed on duplicate SDS-PAGE gels. The protein profiles were visualized by silver staining (A) and gp65 was detected by probing blotted proteins with mAb 4E1. Lane 1, ^{14}C-methylated Rainbow markers (Amersham), 2.5 µl; lane 2, Con A-binding proteins (2.5 µg); lane 3, pooled fractions from DE-52 column used for binding to RCA-agarose (5 µl); lane 4, fraction of proteins not bound to RCA-agarose (5 µl); lane 5, fraction of proteins eluted from RCA-agarose with galactose (5 µl). The position of gp65 is indicated with arrows.


A
B
1
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5
kDa
200-
97.4-
69-
46-
30-
21.5-
14.3-
gp65

5.4 Discussion

To have potential as a target for trypanocidal agents, a molecule must be accessible to externally supplied agents. Since gp65 is a membrane component of an endocytic organelle in *T. vivax*, at least part of it must be oriented on the lumenal face of these organelles for it to come in contact with endocytosed material The experiments carried out in order to investigate the orientation of gp65 in the membrane included its accessibility to different proteases and its ability to be cross-linked in the membrane. Results from the protease protection experiments suggest that gp65 has trypsin cleavage site(s) on the lumenal face of the membrane since digestion of the protein was achieved only in the presence of detergent. gp65 was partially accessible to cleavage by the enzymes endoproteinase Glu-C (SV8) and endoproteinase Asp-N (Asp-N) in the absence of detergent, therefore suggesting the presence of cleavage sites for these enzymes on the cytoplasmic face of membrane vesicles. Since only a proportion of the gp65 molecules appears to be accessible to SV8 and Asp-N in the absence of Triton X-100, but most of the gp65 molecules appear to be accessible to these proteases in the presence of detergent, this could be interpreted to suggest that some of the vesicles (~50%) were already permeabilized or inside out. In these types of experiments, it is common to exploit a molecule of known orientation in the membrane or one that is located within the particular organelle, as a control for intact vesicles. For example, the accessibility of newly synthesized influenza virus haemagglutinin in infected mammalian cells has been used as a control since it is found within the endoplasmic reticulum and Golgi apparatus shortly after synthesis (Narula et al., 1992). With *T. vivax* such convincing control experiments could not be carried out since suitable markers have yet to be established. However, the results from the trypsin experiment argue in favour of intact vesicles, since there was no detectable degradation of gp65 by trypsin until Triton X-100 was added to the incubation mixture, when trypsin was able to cleave all of the gp65 molecules. In an attempt to clarify the protease protection results, cross-linking experiments were carried out using the membrane permeable cross-linker, DSP, and the membrane impermeable cross-linker, DTSSP. It was found that only the membrane permeable reagent cross-linked gp65 in the membrane. Treatment of membranes with DTSSP, the membrane impermeable reagent, failed to cross-link gp65, suggesting that the only accessible NH_2-groups were present on the lumenal side of the organelles. Interpretation of the results from both the protease protection and cross-linking experiments is difficult, but it seems likely that gp65 is a transmembrane protein with trypsin cleavage and NH_2-cross-linking sites on the lumenal face of the vesicles and cleavage sites for Glu-C and Asp-N located on the cytoplasmic side of the vesicles. This does not preclude the possibility that there may be additional cleavage sites for these enzymes on the lumenal face of the membrane. Previous results have shown that both gp65 and p22 were resistant to extraction from membranes with Na_2CO_3 at alkaline pH and that both were enriched by partitioning into Triton X-114 (see section 3.3.3). p22 was assumed to be a proteolytic cleavage product of gp65 that remains associated with the rest of the molecule by disulfide linkage. In order to determine if the p22 domain of gp65 was inserted into the membrane, extraction of membranes with Na_2CO_3 was repeated in the presence of the reducing agent, dithiothreitol (DTT). Since this treatment failed to release p22 into the soluble fraction, it is likely that the p22 portion of the molecule is inserted into the membrane and thus includes a membrane spanning region of gp65.

Studies using limited proteolysis in one dimensional SDS-PAGE gels have been carried out to investigate structural relationships between different proteins (Cleveland, 1983). This approach was used to investigate the relationship between gp65 and p22 since common peptide(s) would be good evidence in support of p22 being a breakdown product of gp65. Two enzymes, SV8 and trypsin, were used at different concentrations to try to digest gp65 and p22 since they had been shown to cleave gp65 in the protease protection experiments. The limitation of this experiment was that the only method available for the detection of cleavage products was western blots probed with the monoclonal antibody. Therefore, peptides that did not contain the epitope recognized by the antibody were undetectable. Digestion of gp65 with SV8 resulted in one detectable digestion product of approximately 20-21 kDa and digestion with trypsin gave one immunoreactive product of 17 kDa. There is a suggestion of an additional SV8 site in the gp65 since the intensity of the band corresponding to the 20-21 kDa cleavage product decreased with increased amounts of enzyme, however, resulting peptide(s) were not detectable. In addition, no detectable cleavage products of p22 were obtained with either enzyme. Therefore, Cleveland mapping did not help to confirm whether p22 is part of the gp65 molecule, but it seems the most likely explanation for the evidence. However, the possibility still exists that p22 is a distinct gene product which shares the mAb 4E1 epitope and is disulfide-linked to gp65.

One dimensional SDS-PAGE gels of *T. vivax* membrane proteins bound and eluted from Con A-Sepharose showed that there were several proteins in the molecular weight range of gp65. Isoelectric focusing and two-dimensional gel electrophoresis with the Con A-binding proteins were carried out in order to try to resolve gp65 from other proteins and to determine if it could be detected by protein staining methods once resolved. Using the ISO-DALT system (Anderson and Anderson, 1978 a, b) ten gels can be run simultaneously which facilitates comparison of resolved proteins detected by different methods. When Con A-binding proteins were resolved using this system, it was found that resolution of proteins was good as judged by protein staining, but gp65 could not be clearly seen in the Coomassie stained gel (Fig. 5.5 A). Similar gels were stained using the more sensitive method of protein detection by silver staining (not shown) but this did not aid in the detection of gp65. By examining the pattern of resolution of the immunoreactive species on the 2-D blot, the gp65 appeared to be composed of two species, one that resolved to five or six spots around the neutral to acidic end of the gel and the other that was found more at the basic end of the gel. The more basic species of gp65 ran slightly slower than the other species, suggesting that it may be of higher molecular weight. The p22 moiety appears to have a fairly acidic isoelectric point (pI). The gp65 contains both N- and O-linked oligosaccharides, therefore the complexity of its resolution pattern could be explained by differential glycosylation of the molecule especially if some glycans contain sialic acid residues. Other post-translational modifications, such as phosphorylation, could also produce the same effect. Differential glycosylation or the possibility of two molecular species of gp65, may also explain the protease protection results, in which only some of the gp65 molecules were accessible to SV8 and endo Asp-N digestion in the absence of detergent. Since gp65 was in its native state in these experiments, it is possible that the tertiary structure of the native glycosylated protein sterically hindered potential protease cleavage sites.

Of note is the immunoreactive species of approximately 100 kDa seen on the 2-D immunoblot (Fig 5.5 B) and in the Coomassie stained gel (Fig. 5.5 A). The 100 kDa spots are aligned above the more neutral gp65 spots. This molecule may be related to gp65 since they are both recognized by mAb 4E1, but whether there is a more significant relationship between the two molecules, for example, a precursor/product, is unknown. The gp65 is much more intensely stained in the immunoblot than the 100 kDa which suggests that the gp65 is present in higher abundance. However, the 100 kDa protein can be stained by Coomassie blue (Fig 5.5 A) whereas gp65 cannot be visualized by protein staining methods. Therefore, either the biochemical properties of gp65, glycosylation for example, prevent it from being easily detected by these staining methods or the 100 kDa is present in greater abundance and contains a weakly cross-reactive mAb 4E1 epitope.

Immunoaffinity purification of gp65 was not successful. It appears that gp65, in its native state, cannot bind to mAb 4E1 since the antibody did not work in ELISA or dot blot assays. Therefore to obtain gp65 in a more purified form so that limited amino-terminal sequence analysis could be attempted, the Con A-binding proteins which were enriched in gp65 were separated by DE-52 cellulose chromatography. Since gp65 was detected in fractions that eluted from the column before the fractions containing the majority of protein, an enrichment of gp65 was obtained. Further enrichment of gp65 was achieved by binding pooled gp65-containing DE-52 fractions to RCA-agarose. Although, not striking on the silver stained SDS-PAGE gel, there was an enrichment of a band in the molecular weight range of 65 kDa which may correspond to gp65. However, the enrichment was evident on the immunoblot since there was an increase in intensity of the gp65 band in the fraction eluted from RCA agarose. Similar samples to the one depicted in Fig. 5.8 A (lane 4), were separated on SDS-PAGE gels, blotted onto Immobilon-P™ (Matsudaira et al., 1987), excised and amino-terminal sequence analysis was attempted at the University of Victoria. Three separate samples were sent and no sequence was obtained, either due to a low quantity of gp65 or a chemical blockage of the N-terminus.

In summary, results presented in chapters 3 and 5 indicate that gp65 is an integral membrane protein, most probably of transmembrane nature with a membrane spanning region within the p22 portion of the molecule. gp65 appears to exist partially as oligomeric complexes, held together by disulfide linkages. As judged by the resolution pattern of gp65 on two-dimensional gels, it appears to be a complex protein, possibly existing in more than one physical state which may be due to post-translational modifications. Evidence of different types of glycosylation has been established in this study but other modifications, such as phosphorylation or fatty acylation, which may also contribute to the complex pattern seen on the two-dimensional gels have not been characterized. Therefore, this protein appears to be a very complex molecule in terms of its structural organization. Since no sequence data could be generated for gp65, its function in the endocytic pathway of *T. vivax* remains speculative. If gp65 contains a cytoplasmic domain, this feature could indicate a possible function for the protein. The cytoplasmic domain of some integral membrane proteins have been shown to be involved in recognition and binding of other proteins in order to facilitate their function. For example, the cytoplasmic domain of the LDL-receptor (Davis et al., 1986) and the transferrin receptor (Jing et al., 1990) contain sequences which are necessary for receptor internalization via coated pits. The lysosomal membrane glycoproteins (lgp's) are transmembrane proteins with small cytoplasmic tails (Fukuda, 1991). The conserved tetrapeptide (His-Ala-Gly-Tyr) found in the cytoplasmic tail of lgps is thought to be necessary for targeting of these molecules since it enabled a reporter molecule to be directed to lysosomes (Williams and Fukuda, 1990). In addition, the tyrosine residue appears to be crucial for targeting of the lgp to the lysosome which is analogous to the role of tyrosine in the cytoplasmic domain of the transferrin and LDL-receptors.

By analogy, the putative cytoplasmic domain of the gp65 could function in the targeting of the protein to the endocytic organelles. Alternatively, it could function in the recognition and binding of cytoplasmic proteins that mediate fusion and fission events. An example of such proteins are the rab proteins, a family of small GTP-binding proteins which are associated with specific compartments in the endocytic and exocytic pathways of mammalian cells (Zahraoui et al., 1989; Chavrier et al., 1990). Small GTP-binding proteins are thought to regulate the transfer of membrane between specific transport stations (Bourne, 1988; Mayorga et al., 1989). Perhaps gp65 functions in binding rab-like proteins that in turn mediate fusion between endocytic vesicles or promote fission to allow recycling of receptors or other molecules.

Further characterization of gp65 is necessary to gain further insight into its function in the endocytic pathway of *T. vivax*. It would be interesting to obtain a full length DNA sequence so that any homology to known molecules could be assessed and to determine if a transmembrane region and cytoplasmic domain are predicted by the sequence. Further, it would be interesting to determine if there is a peptide signal sequence similar to those described for lysosomal membrane glycoproteins or present in the cytoplasmic domain of gp65. If no such sequences could be found the localization of gp65 expressed in transfected mammalian cells could be determined which might provide a better indication of the function of this protein.

CHAPTER 6

Other Invariant Antigens of *T. vivax*

6.1 Introduction

During preliminary studies on the invariant antigens of *T. vivax*, attempts were made to isolate a subcellular fraction which was highly enriched in surface membranes. Since 3'-nucleotidase activity is located on the surface membrane of *T. b rhodesiense* (Gardiner et al., 1982; Gottlieb et al., 1986), similar enzymatic activity was sought in *T. vivax*, as it had never been shown to exist in this species Although a better method for the enrichment of plasma membranes was not achieved, 3'-nucleotidase activity, which corresponded to a molecule with an approximate molecular mass of 35 kDa, was found to be associated with both membrane and soluble fractions of *T. vivax*.

Monoclonal antibodies were raised to formalin-fixed, uncoated forms of *T. vivax* and several were characterized to some degree as discussed in Chapter 2 One of the monoclonal antibodies, mAb 4B11, an IgM, identified a 35 kDa antigen which was partially localized to the surface of the cells. Partial characterization of two invariant molecules of *T. vivax*, the 3'-nucleotidase and the 35 kDa mAb 4B11-reactive protein is discussed in this chapter.

6.2 Materials and Methods

6.2.1 Tube assay for 3'-nucleotidase activity.

Freshly isolated bloodstream forms of *T. vivax* ILDat 2.1 were lysed by two different methods. First, 1 x 10^8 parasites/ml were solubilized on ice in the following buffer: 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.5% (v/v) Triton X-100 containing 50 µg/ml each of antipain, E-64, and leupeptin. Cells were frozen in liquid nitrogen and thawed at 37°C (three cycles), homogenized for 2 minutes with a tight fitting glass homogenizer, then centrifuged at 120,000*g* for 1 hour at 4°C. The supernatant, containing the soluble fraction, was removed from the insoluble pellet and kept on ice. The pellet was resuspended by homogenization in ice-cold buffer and adjusted to the original volume. Assays of 3'-nucleotidase activity were performed using equivalent aliquots from each fraction. This method was not quantitative in terms of a comparison of activities in different fractions on an equal protein basis. However, since the volumes were the same, the relative activity in each fraction could be compared in a qualitative manner. 100 µl of sample was added to 400 µl of the incubation buffer: 50 mM Tris-maleate (pH 8.5), 100 mM KCl, 1 mM $CoCl_2$, and 2.5 mM 3'AMP as substrate (Gottlieb et al., 1986) and incubated at 37°C for 30 minutes. For the measurement of inorganic phosphate, the product of the 3'-nucleotidase reaction, 0.1 ml of a freshly prepared solution of 60% trichloroacetic acid was added to 500 µl of reaction mixture and centrifuged for 10 minutes at 20,000*g* in an Eppendorf centrifuge. 500 µl of the resultant supernatant was removed from the tubes after centrifugation and placed in a clean test tube. 2 ml of developing reagent (1 g ascorbic acid in 10 ml dH_20, added to 50 ml 0.342 g K/Na molybdate in 1N H_2SO_4) was added to each tube and incubated at 50°C for 20 minutes (Gottlieb et al., 1986). After the tubes had cooled, the absorbance was read in a spectrophotometer at 820 nm. Controls for this experiment consisted of 100 µl samples incubated in the same buffer without substrate. Blanks consisted of buffer alone (100 µl of lysis buffer plus 400 µl incubation buffer) with the added detection reagent.

3'-nucleotidase activity was also measured in the soluble and membrane fractions of *T. vivax*, after hypotonic lysis, as follows. Cells were suspended at a concentration of 1 x 10^8 cells/ml in 10 mM Tris-HCl (pH 8.0), 2 mM EDTA containing 50 µg each of antipain, E-64, and leupeptin, and disrupted by homogenization for 5 minutes on ice, using a tight fitting Dounce homogenizer. Insoluble material was separated from the soluble fraction by centrifugation at 120,000*g*. The soluble proteins were removed and the pellet was resuspended in the starting volume of the same buffer. To reduce non-specific protein adsorbance to the dialysis tubing, 0.01% (v/v) Triton X-100 was added to each sample and dialyzed against 4 litres of 10 mM Tris-HCl (pH 8.0), overnight at 4°C. After dialysis, part of each fraction was adjusted to 1 mM EDTA and the other part received no EDTA. 3'-nucleotidase activity of these samples was assayed as described above, in the presence and absence of 1 mM $CoCl_2$.

To determine the effect of tartrate on *T. vivax* 3'-nucleotidase activity, the dialyzed soluble fraction, obtained after hypotonic lysis and homogenization, was incubated with varying concentrations of potassium tartrate and the level of 3'-nucleotidase activity was assayed.

6.2.2 Gel assay for the *in situ* detection of 3'-nucleotidase.

Protein samples were separated by SDS-PAGE under reducing conditions (Laemmli, 1970), and 3'-nucleotidase activity was detected by the malachite green/molybdate method for release of inorganic phosphate (Zlotnick and Gottlieb, 1986; Zlotnick et al., 1987). Briefly, after electrophoresis, gels were washed in 0.1M Hepes-NaOH (pH 8.5) with 0.1% (w/v) CHAPSO using 3 changes of 100 ml of buffer (30 minutes each wash) with shaking. The fourth wash was carried out overnight at 4°C and the next day, the gel was incubated for 30 minutes at 37°C in fresh buffer containing 2.5 mM 3'-AMP. Following incubation with substrate, the gel was rinsed with distilled H_2O to remove excess phosphate from the surface of the gel, then incubated in the diluted phosphate detection reagent as described (Zlotnick and Gottlieb, 1986). Gels were photographed immediately after the colour reaction occurred, since background development was rapid.

6.2.3 Production of mAb 4B11.

mAb 4B11 was produced as described in section 3.2.3. It was selected after screening tissue culture supernatants by western blot and immunolocalization on thin cryosections of bloodstream form *T. vivax* (see sections 3.2.5 and 3.2.7 respectively for details). mAb 4B11 tissue culture supernatant was diluted 1:10 for use on western blots and 1:50 for labelling of cryosections.

6.3 Results

6.3.1 *T. vivax* 3'-nucleotidase activity is partially soluble and inhibited by EDTA.

Initial determinations of 3'-nucleotidase activity in the Triton X-100 soluble and insoluble fractions of *T. vivax* using the tube assay, demonstrated that most of the 3'-nucleotidase activity was present in the detergent soluble fraction and very little was left in the insoluble pellet (Table 6.1). In addition, it can be seen that the control samples (no added 3'-AMP) contained a high level of endogenous organic phosphate and the absorbance values are corrected to yield the net absorbance at 820 nm.

Table 6.1

***T. vivax* 3'-nucleotidase activity is associated with the detergent soluble fraction.**

Fraction	3'-AMP	Control	Net A_{820}
Triton X-100	2.170	1.360	0.810
Soluble (100 μl)	2.244	1.538	0.706
	1.971	1.326	0.645
		$\bar{x}$ =	0.720
Triton X-100	0.211	0.050	0.161
Insoluble	0.208	0.062	0.146
(100 μl)	0.136	0.019	0.117
		$\bar{x}$ =	0.141

Previous studies of 3'-nucleotidase activity in *T. b. rhodesiense*, demonstrated that although the activity was membrane associated, much of it could be released into the supernatant fraction by lysing cells in hypotonic buffer without detergent (Gottlieb et al., 1986). This procedure was tested with *T. vivax* to determine whether the 3'-nucleotidase could be similarly solubilized. Results presented in Table 6.2 demonstrate that most of the 3'-nucleotidase activity is associated with the supernatant fractions. This can be seen best in the samples with no added EDTA. Secondly, addition of 1 mM EDTA reduced 3'-nucleotidase activity to less than half the value obtained when no EDTA was added. This inhibition could be restored by the addition of an equimolar concentration of $CoCl_2$. In addition, it appears that Co^{++} ions slightly enhanced the enzyme activity in the absence of EDTA.

Table 6.2

Effect of EDTA and Co^{++} on 3'-nucleotidase activity

Fraction	**Co^{++}**	**+ EDTA**	**- EDTA**
sup	-	0.078*	0.202
pellet	-	0.021	0.097
sup	+	0.278	0.271
pellet	+	0.101	0.140

* Average of 3 determinations; corrected for control values.

Results from the tartrate inhibition experiments are presented in Table 6.3. These results show that potassium tartrate has no inhibitory effect on 3'-nucleotidase activity.

Table 6.3

Effect of Tartrate on 3'-nucleotidase activity

[Tartrate]	**A_{820}**
0 mM	0.435*
2 mM	0.437
10 mM	0.408

* Average of 3 determinations; corrected for control values.

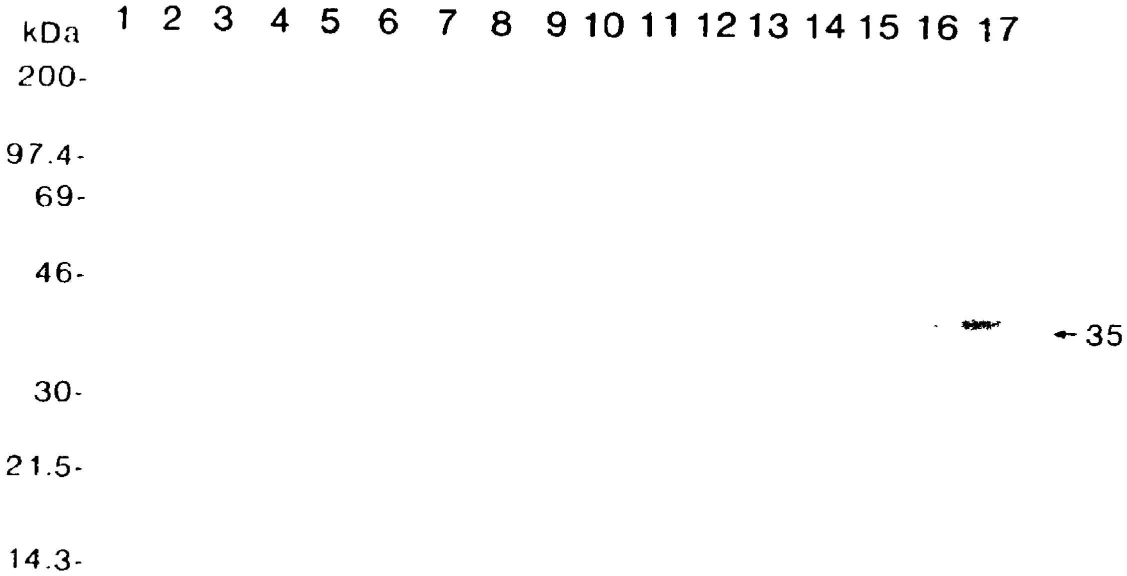
6.3.2 The *T. vivax* 3'-nucleotidase has a molecular mass of 35 kDa.

Aliquots from various subcellular fractions were obtained following disruption of cells in an isotonic buffer and sucrose density centrifugation (experiment described in section 3.3.3 A). Proteins were separated on an SDS-PAGE gel and 3'-nucleotidase activity was detected by the *in situ* gel method. 3'-nucleotidase activity, corresponding to a molecular mass of 35 kDa, was found in all the membrane fractions obtained from the differential centrifugation steps (Fig. 6.1, lanes 2-5) and a substantial amount was also present in the 120,000*g* supernatant which represents the soluble fraction (Fig. 6.1, lane 6). When the 120,000*g* pellet was resuspended and

Figure 6.1

3'-nucleotidase activity in subcellular fractions of *T. vivax*.

SDS-PAGE gel stained by the malachite green method for detection of phosphate to visualize the presence and approximate molecular mass of the 3'-nucleotidase activity. Lanes 1-6 contain starting material and fractions from the differential centrifugation steps (25 ug protein/lane). Lane 1, *T. vivax* ILDat 2.1 bloodstream forms; lane 2, 750g pellet; lane 3, 4,500g pellet; lane 4, 30,000g pellet, lane 5, 120,000g pellet; lane 6, 120,000g supernatant. Lanes 7-17 contain aliquots from selected fractions from the sucrose density gradient (50 ul from each fraction). Lane 7, fraction 2; lane 8, fraction 4; lane 9, fraction 6; lane 10, fraction 9; lane 11, fraction 11; lane 12, fraction 13; lane 13, fraction 16; lane 14, fraction 18; lane 15, fraction 20; lane 16, fraction 24; lane 17, fraction 26.


kDa
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
200-
97.4-
69-
46-
30-
21.5-
14.3-
35

centrifuged through a sucrose density gradient, as described in section 3.2.9, enzyme activity was found in all fractions which had a detectable amount of protein (Fig. 6.1, lanes 7-17), with a slight enrichment found in the highest density fraction (Fig. 6.1, lane 17).

6.3.3 Extraction of the 3'-nucleotidase with Na_2CO_3 and partitioning into the detergent phase of Triton X-114.

Aliquots from fractions resulting from Na_2CO_3 extraction and Triton X-114 phase separation of membrane proteins (described in sections 3.2.11 and 3.2.12), were separated on an SDS-PAGE gel, renatured and stained for 3'-nucleotidase activity. Results show that after Na_2CO_3 extraction of membranes, most of the 3'-nucleotidase activity was seen in the supernatant fraction (Fig. 6.2, lane 1) and little was left in the pellet (Fig. 6.2, lane 2). After partitioning into Triton X-114, most of the 3'-nucleotidase activity was detected in the detergent phase (Fig. 6.2, lane 4) and very little was found in the aqueous phase (Fig. 6.2, lane 3).

6.3.4 3'-nucleotidase binds to Concanavalin A-Sepharose.

3'-nucleotidase activity was present in the fraction of proteins that bound to and were specifically eluted from Con A-Sepharose with mannose (see section 3.2.13) (Fig 6.3, lane 2). No activity was observed in the fraction containing proteins which did not bind to the lectin (Fig 6.3, lane 1).

Figure 6.2

The *T. vivax* 3'-nucleotidase can be extracted from the membrane with Na_2CO_3 but partitions into the detergent phase of Triton X-114.

SDS-PAGE gel of the supernatant (lane 1) and pellet (lane 2) fractions of membranes treated with 0.2 M Na_2CO_3 and the aqueous (lane 3) and detergent (lane 4) phases of Triton X-114 after partitioning. The gel was stained for liberated phosphate to detect 3'-nucleotidase activity. 25 μg protein loaded in each lane.

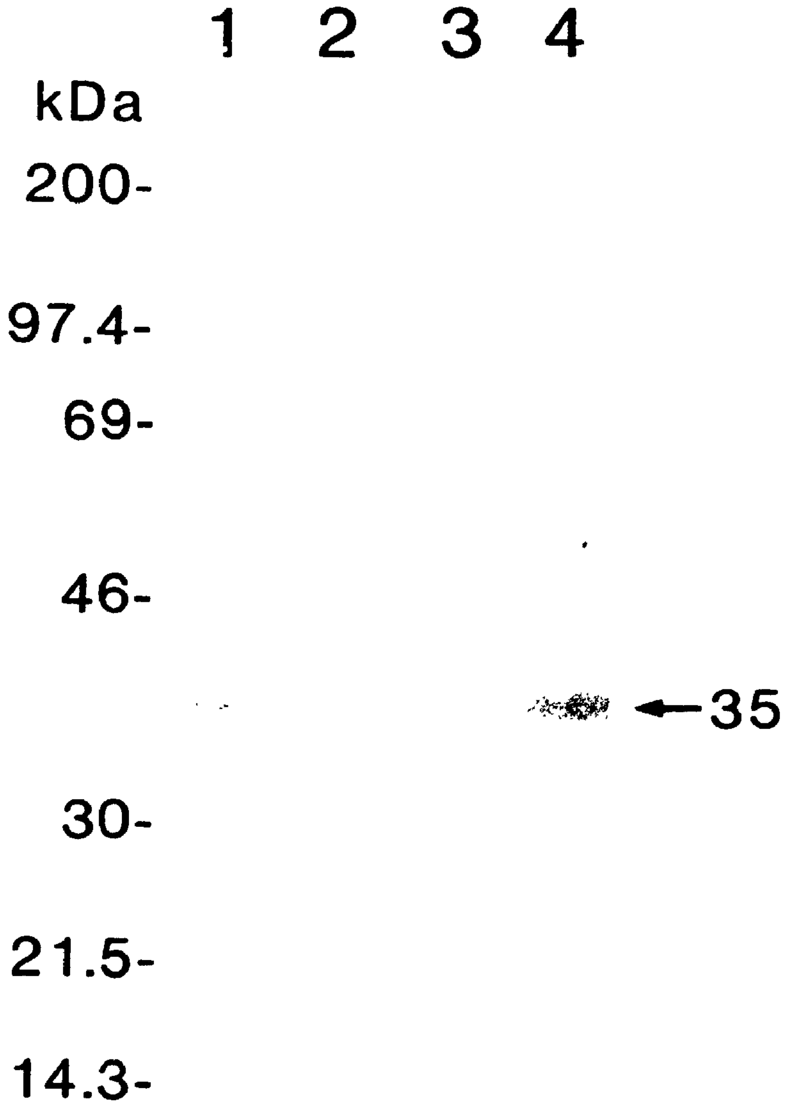
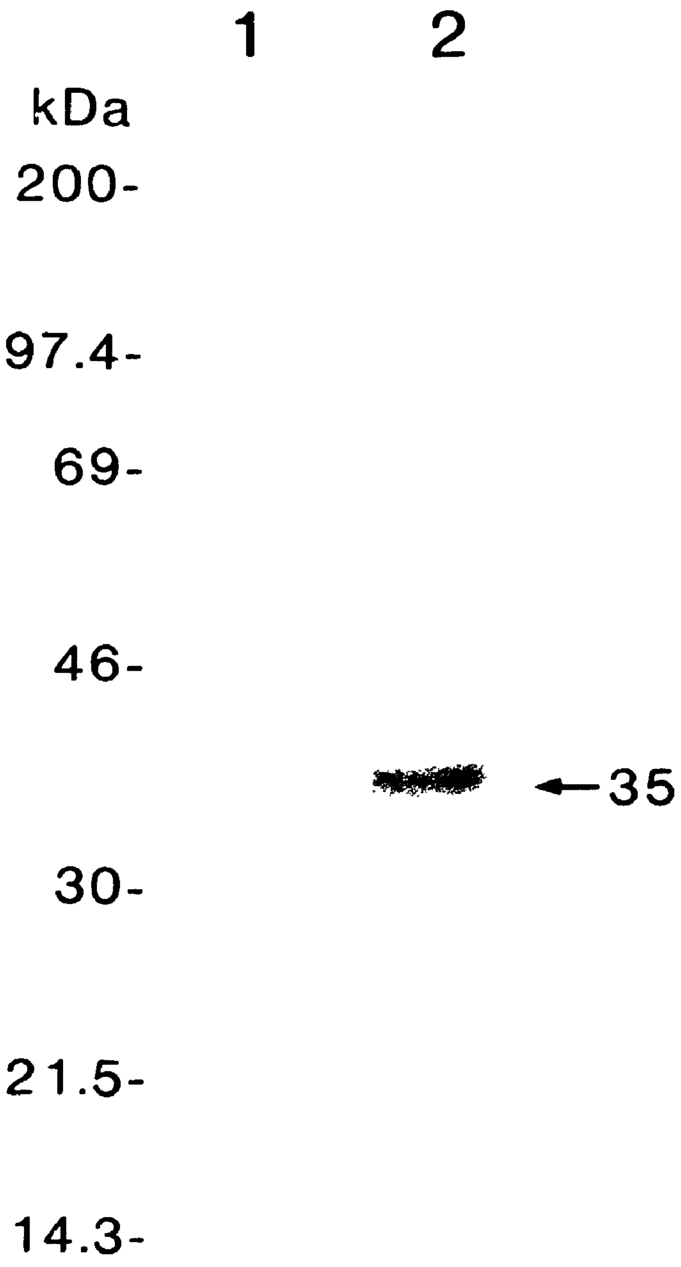

1
2
3
4
kDa
200-
97.4-
69-
46-
←35
30-
21.5-
14.3-

Figure 6.

3'-nucleotidase binds to Concanavalin A-Sepharose.

The *T. vivax* 3'-nucleotidase was enriched in a fraction of total solubilized membrane proteins that bound to ConA-Sepharose (lane 2), but no activity was detected in the ConA-Sepharose unbound fraction (lane 1). 25 µg total protein loaded per lane.


1
2
kDa
200-
97.4-
69-
46-
35
30-
21.5-
14.3-

6.3.5 Specificity of mAb 4B11.

On western blots, mAb 4B11 identified a 35 kDa protein in bloodstream forms of *T. vivax* ILDat 1.2 (Fig. 6.4, lane 1). mAb 4B11 reactivity was specific to *T. vivax* as neither bloodstream forms of *T. brucei* or *T. congolense* (Fig. 6.4, lanes 2 and 4), nor procyclic forms of *T. brucei* (Fig. 6.4, lane 3) were recognized by the antibody. The 35 kDa antigen was not included among the proteins eluted from Con A-Sepharose (Fig. 6.4, lane 5). Most of the 35 kDa molecule appears to be membrane-associated since it was enriched in the membrane pellet after centrifugation of post-nuclear supernatant at 120,000*g* (Fig. 6.4, lane 7) and little was released into the soluble fraction (Fig. 6.4, lane 6).

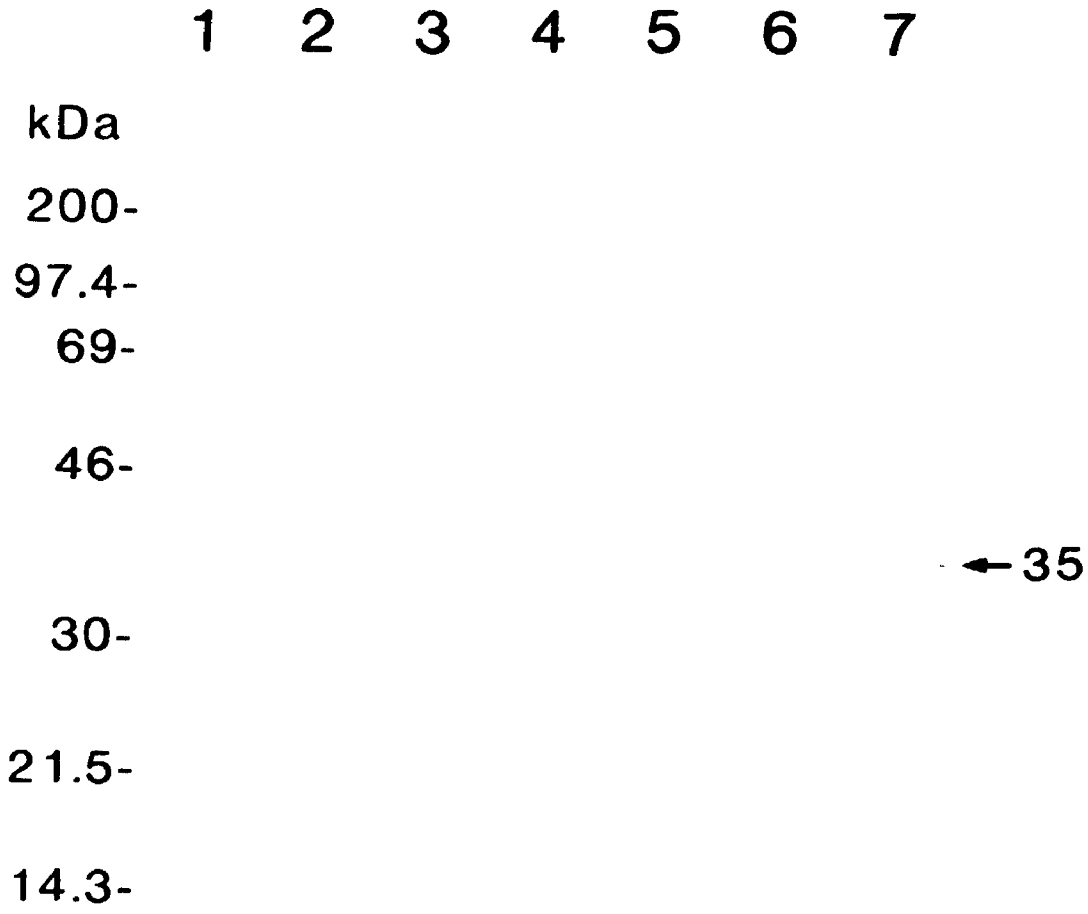
6.3.6 Immunolocalization of the 35 kDa invariant antigen.

The use of mAb 4B11 to label ultrathin cryosections of bloodstream form *T. vivax* revealed that much of the label was associated with the surface of the cell and the flagellum (Fig. 6.5, indicated by arrowheads). However, internal labelling as well as labelling within the lumen of a membrane-bound structure, possibly the flagellar pocket, was also observed.

Figure 6.4

Specificity of mAb 4B11.

Western blot probed with mAb 4B11 and visualized with 2 uCi ^{125}I-anti mouse Ig. Lane 1, *T. vivax* ILDat 2.1 bloodstream forms; lane 2, *T. brucei* GuTat 3.1 bloodstream forms; lane 3, *T. brucei* GuTat 3.1 procyclic forms; lane 4, *T. congolense* bloodstream forms; lane 5, total solubilized membranes from *T. vivax* bloodstream forms bound to ConA-Sepharose; lane 6, 120,000g supernatant; lane 7, 120,000g pellet. 25 µg protein loaded in each lane.


1
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kDa
200-
97.4-
69-
46-
←35
30-
21.5-
14.3-

Figure 6.5

Immunolocalization of the 35 kDa invariant antigen on thin cryosections of *T. vivax* ILDat 2.1.

Ultrathin cryosections, thawed and labelled with mAb 4B11 tissue culture supernatant at 1:10 dilution. Second antibody labelling was achieved using a rabbit anti-mouse Ig, followed by 10 nm protein A-gold. Arrowheads indicate surface labelling; flagellum (f); nucleus (n). Scale bar = 0.25 µm.

detected. A cysteine-rich transmembrane protein of predicted molecular mass 130 kDa, referred to as CRAM, has been identified by the sequencing of a cloned *T. brucei* cDNA (Lee et al., 1990). Antibodies raised in rabbits to a short fusion protein were used to localize CRAM on thin sections of Lowicryl-embedded bloodstream form and procyclic form trypanosomes. The authors reported that the protein was localized to the flagellar pocket and within the lumen of vesicular organelles. The expression of this protein was much higher in procyclic forms of the parasite than in bloodstream forms. Although CRAM has sequence homology to the human LDL-receptor and is certainly located in an appropriate position to mediate the internalization of LDL, it is doubtful that CRAM is related to the LDL receptor purified from bloodstream form parasites due to differences in stage expression and molecular mass.

Using antibodies to the mammalian epidermal growth factor (EGF) receptor, a homologous protein of 135 kDa has been identified in bloodstream and procyclic forms of *T. brucei* (Hide et al., 1989). Sequence analysis of a cDNA from a *T. brucei* library identified an open reading frame which had strong homology to the human glucose transporter gene (Bringaud and Baltz, 1992). Transfection of *Xenopus* oocytes defective for hexose transport with the *T. brucei* cDNA produced cells that were capable of taking up glucose (T. Baltz, personal communication). Recently, two invariant surface glycoproteins of *T. brucei* have been identified and characterized (Ziegelbauer and Overath, 1992; Ziegelbauer et al., 1992). These proteins, designated

6.4 Discussion

6.4.1 Two new invariant membrane antigens of *T. vivax*.

The initial phase of this project was concerned with the identification of invariant surface antigens of *T. vivax*. The first approach taken was to try to generate a subcellular fraction that was enriched in surface membranes. It was anticipated that this fraction would then be used to immunize rabbits in order to raise polyclonal antisera for the purpose of isolating specific invariant antigens. To isolate plasma membranes of *T. vivax*, the protocol of Rovis and Baekkeskov (1980; which was developed for the isolation of *T. brucei* membranes) was followed. To assess the degree of purification of surface membranes, it was necessary to a follow the enrichment of a known plasma membrane marker. Typically, 5'-nucleotidase has been employed as a marker in purification schemes, since it is associated with the plasma membrane of a variety of cell types (DePierre and Karnovsky, 1973; Trams and Lauter, 1974). Although 5'-nucleotidase activity was reported to be present in *T. brucei* (Voorheis et al., 1979), more recent reports indicate that trypanosomes do not have a 5'-nucleotidase (Rovis and Baekkeskov, 1980; McLaughlin, 1982; Gottlieb et al., 1986).

3'-nucleotidase, although not usually found in mammalian cells (Drummond and Yamamoto, 1971), is associated with the surface membranes of several protozoan parasites, *Leishmania donovani* (Gottlieb and Dwyer, 1983), *Crithidia fasciculata* (Gottlieb, 1985), *Entamoeba histolytica* (Hassan and Coombs, 1986) and *Trypanosoma brucei rhodesiense* (Gardiner et al., 1982; Gottlieb et al., 1986). The best characterized 3'-nucleotidases are the leishmanial and crithidial enzymes. These enzymes appear to be closely related as they exhibit similar characteristics. They both have an apparent molecular mass of 43 kDa, they are able to hydrolyze nucleic acids as well as 3'-ribonucleotides and the enzyme activity is inhibited by EDTA which can be reversed by the addition of cobalt ions (Gottlieb and Zlotnick, 1987; Gottlieb, 1989). In addition, the expression of the 3'-nucleotidase has been shown to be inducible upon purine starvation (Gottlieb, 1985; Sacci et al., 1990). Only the *Crithidia luciliae* enzyme has been purified so far (Neubert and Gottlieb, 1990). Further characterization of the purified enzyme will facilitate the study of the control of gene expression, once the DNA sequence has been obtained. The *T. b. rhodesiense* 3'-nucleotidase has not been characterized to such an extent, but its surface localization has been established by cytochemical techniques (Gottlieb et al., 1986). Although 3'-nucleotidase had never been demonstrated in *T. vivax* the possibility existed that it would provide a potential enzymatic marker to follow the purification of surface membranes since it was present in the closely related species, *T. brucei*. Results from 3'-nucleotidase assays using detergent solubilized proteins clearly show that *T. vivax* bloodstream forms possess 3'-nucleotidase activity (Table 6.1). Results presented (Table 6.2) show that after lysis of bloodstream forms of *T. vivax* in a hypotonic buffer, most of the 3'-nucleotidase activity was associated with the soluble fraction. This result is consistent with results obtained in previous studies of the *T. brucei* 3'-nucleotidase (Gottlieb et al., 1986). When cells were lysed by physical disruption in isotonic medium, as they were for the subcellular fractionation experiment (Fig. 6.1), some of the 3'-nucleotidase activity was released into the soluble fraction, although most remained associated with membrane fractions. This result differs from that of McLaughlin (1982) where it was reported that the *T. brucei* 3'-nucleotidase remained particle-associated after disruption of cells in isotonic medium. In addition to its solubilization properties, the *T. vivax* enzyme was shown to be inhibited by EDTA and this inhibition could be overcome by the addition of cobalt ions (Table 6.2). Furthermore, the commonly used phosphatase inhibitor, potassium tartrate, had no affect on enzyme activity (Table 6.3). Therefore, the characteristics of the *T. vivax* 3'-nucleotidase are similar to those previously described for the 3'-nucleotidases of *L. donovani* and *C. luciliae* (Gottlieb, 1989).

Enzyme activity in subcellular fractions obtained from sucrose density gradient centrifugation were monitored by *in situ* detection in SDS-PAGE gels. It was performed in order to assess whether this method would provide a better means to follow enrichment of enzyme activity in specific fractions. Results from this experiment, shown in Fig. 6.1, demonstrate that there was no enrichment of 3'-nucleotidase in any particular fraction, except the highest density fraction. This apparent lack of enrichment of 3'-nucleotidase can be explained if the enzyme is not found exclusively on the surface of the cells. The surface association of the *T. vivax* enzyme had not been established by experimental means but the enzyme was assumed to be located on the surface by virtue of the fact that the other trypanosomatid 3'-nucleotidases are cell surface-associated. It is also possible that some of the surface membrane may remain attached to the pellicular microtubules thus enzyme activity was found in some of the higher density fractions. A better technique for isolation of plasma membrane of *T. vivax* is needed.

The ***in situ*** gel assay proved to be a better method for 3'-nucleotidase activity since it was simpler to perform, and the molecular mass of the enzyme could be determined simultaneously. The apparent molecular mass of the *T. vivax* 3'-nucleotidase, 35 kDa, was found to be lower than that determined for *L. donovani* and *C. luciliae*, 43 kDa (Gottlieb, 1989). The possibility exits that the 35 kDa band seen in *T. vivax* fractions is a functional proteolytic cleavage product of a larger molecule. However, the molecular weight of the *T. brucei* enzyme was also found to be 35 kDa in this study by the same assay (not shown) thus perhaps the trypanosomal enzymes have a sensitive proteolytic cleavage site not found in the enzymes of the other species.

Since the leishmanial enzyme is a glycoprotein with N-linked oligosaccharides (Sacci et al., 1990) and the *T. vivax* 3'-nucleotidase is a putative membrane protein, by analogy, it may also be a glycoprotein. Therefore, detergent-solubilized membrane proteins of *T. vivax* were bound to and eluted from Con A-Sepharose, and the 3'-nucleotidase activity was examined in both the bound and unbound fractions. The results show that all of the 3'-nucleotidase activity was associated with the fraction that bound to the lectin, suggesting that the *T. vivax* 3'-nucleotidase is a glycoprotein.

The potential membrane-association of the 3'-nucleotidase activity was addressed by determining the ability to extract the enzyme from membranes using Na_2CO_3 at alkaline pH and its behaviour following Triton X-114 phase separation of

T. vivax proteins (Fig. 6.3). The results show that the 3'-nucleotidase was released from the membrane fraction after incubation with Na_2CO_3. The capacity of proteins to be extracted with Na_2CO_3 usually suggests that the protein is loosely associated with membranes in a non-covalent fashion *i.e.* it is either a peripheral membrane protein, or a soluble protein which is normally located within organelles, or which has become trapped within vesicles which reformed after disruption of the cells (Fujiki et al., 1982). These results, coupled with the release of the 3'-nucleotidase when parasites were lysed in hypotonic buffer, suggest that the enzyme is not tightly associated with the membrane. The results from the Triton X-114 experiment contradict these results since most of the enzyme activity partitioned into the detergent phase, a property usually exhibited by integral membrane proteins. It is possible that the *T. vivax* 3'-nucleotidase is non-covalently associated with an integral membrane protein on the surface and remains associated with this protein upon detergent solubilization and segregation into the detergent phase of Triton X-114.

When surface membranes, prepared by the method of Rovis and Baekkeskov (1980), were used to immunize rabbits for the production of polyclonal antisera, a predominantly anti-VSG response was induced (not shown). Therefore, an alternative approach was adopted in order to identify invariant surface membrane proteins of *T. vivax*. To this end, monoclonal antibodies were raised against whole, fixed, uncoated (no VSG coat) forms of *T. vivax*. Only one of the monoclonal antibodies obtained, mAb 4B11, was directed to an invariant surface molecule and thus it was chosen for use in further characterization of the antigen. mAb 4B11 identified a

35 kDa antigen in all lysates of *T. vivax* tested (not shown) in a species-specific manner since it did not recognize any antigens in *T. brucei* or *T. congolense*. Although mAb 4B11 reacted weakly in immunofluorescence (not shown), it gave satisfactory labelling on ultrathin cryosections of *T. vivax* bloodstream forms (Fig. 6.5). Using this method of immunolocalization, the 35 kDa antigen was primarily localized to the surface of the cells but there also appeared to be a substantial amount localized internally. mAb 4B11 reacted with an antigen that had the same apparent molecular mass as the 3'-nucleotidase, 35 kDa, and was localized to the cell surface, where the 3'-nucleotidase was assumed to be located. The possibility exists that mAb 4B11 was specific for the 3'-nucleotidase, since they exhibited similar properties: molecular weight, surface localization, and membrane association with a proportion released into the soluble fraction. However, the mAb 4B11-reactive antigen did not bind to Con A-Sepharose whereas most of the 3'-nucleotidase activity was found in the fraction of proteins that had bound to the lectin. To investigate this more fully, a better characterization of both proteins would be needed. Unfortunately, the mAb 4B11 hybridoma stopped secreting active antibody which terminated the immediate comparison of the two molecules.

6.4.2 Invariant membrane antigens of trypanosomes.

Recent interest in the invariant surface membrane proteins of bloodstream form trypanosomes has emerged from two not unrelated perspectives. Firstly, since little is known about the proteins of the cell surface that are expected to act as receptors for endocytosis, transporters of nutrients, or other regulators of cellular function, these aspects of the cell biology of the parasites are not well understood. Secondly, interest lies in the potential of invariant proteins to serve as targets for disease control either by immunoprophylaxis or chemotherapy. The trypanosome cell surface is of particular interest as it is assumed to be totally covered with a tightly packed protein coat (Cross, 1975) and putative invariant membrane proteins would appear to be masked. However, since ligands such as transferrin and low density lipoprotein are able to bind their putative receptors leading to receptor-mediated endocytosis (Coppens et al., 1987; Coppens et al., 1991), the barrier imposed by VSG cannot be impenetrable.

Several invariant membrane proteins of bloodstream form trypanosomes have been identified by different means. Crude membrane fractions enriched in plasma membranes (Rovis et al., 1984) or flagellar pocket membrane (McLaughlin, 1987; Olenick et al., 1988) have been used to immunize animals for the purpose of inducing a protective immune response. Although the results were variable, in general, this approach did not succeed in protecting immunized animals against disease. An 86 kDa LDL-receptor has been purified from bloodstream form *T. brucei* and monospecific polyclonal antisera have been used to localize this protein to the flagellar pocket and membrane of the flagellum (Coppens et al., 1988, 1991). No binding was observed on the cell surface of the trypanosome. Since fixed, non-permeabilized trypanosomes were used for immunogold labelling, organelles such as coated vesicles or endosomes which should contain internalized receptor could not be detected. A cysteine-rich transmembrane protein of predicted molecular mass 130 kDa, referred to as CRAM, has been identified by the sequencing of a cloned *T. brucei* cDNA (Lee et al., 1990). Antibodies raised in rabbits to a short fusion protein were used to localize CRAM on thin sections of Lowicryl-embedded bloodstream form and procyclic form trypanosomes. The authors reported that the protein was localized to the flagellar pocket and within the lumen of vesicular organelles. The expression of this protein was much higher in procyclic forms of the parasite than in bloodstream forms. Although CRAM has sequence homology to the human LDL-receptor and is certainly located in an appropriate position to mediate the internalization of LDL, it is doubtful that CRAM is related to the LDL receptor purified from bloodstream form parasites due to differences in stage expression and molecular mass.

Using antibodies to the mammalian epidermal growth factor (EGF) receptor, a homologous protein of 135 kDa has been identified in bloodstream and procyclic forms of *T. brucei* (Hide et al., 1989). Sequence analysis of a cDNA from a *T. brucei* library identified an open reading frame which had strong homology to the human glucose transporter gene (Bringaud and Baltz, 1992). Transfection of *Xenopus* oocytes defective for hexose transport with the *T. brucei* cDNA produced cells that were capable of taking up glucose (T. Baltz, personal communication). Recently, two invariant surface glycoproteins of *T. brucei* have been identified and characterized (Ziegelbauer and Overath, 1992; Ziegelbauer et al., 1992). These proteins, designated

ISG65 and ISG75 were specifically expressed in bloodstream forms and higher molecular weight cross-reactive proteins were detected in lysates of *T. vivax* and *T. congolense* by western blot analysis. However, these surface antigens do not appear to be accessible to antibody since neither anti-ISG65 or ISG75 antisera bound to live trypanosomes. Of the invariant surface membrane proteins of trypanosomes which have been identified, no information exists on the potential for these proteins to induce a protective immune response in animals, apart from a few studies which had variable degrees of success (Rovis et al., 1984; McLaughlin, 1987; Olenick et al., 1988).

In summary, *T. vivax* has been shown to possess a 35 kDa 3'-nucleotidase which is inhibited by EDTA but not by potassium tartrate. The inhibition of activity by EDTA can be overcome by the addition of cobalt ions. The enzyme is readily solubilized with 0.5% Triton X-100 and by hypotonic lysis of cells and a substantial amount of enzyme can be released by physical disruption in isotonic buffer. The 3'-nucleotidase can be extracted from membranes using sodium carbonate at alkaline pH, but it also exhibits properties of a hydrophobic protein since it segregates into the detergent phase of Triton X-114. Although the surface localization of the *T. vivax* 3'-nucleotidase has not been established, it is assumed to be located at the surface of these cells since it has been shown to be surface-associated in the closely related trypanosome species *T. brucei* as well as in other protozoan parasites such as *L. donovani*, *C. luciliae*, and *E. histolytica*. The mAb 4B11, an IgM, which was raised to whole, fixed, uncoated, culture forms of *T. vivax* recognized a 35 kDa antigen that was predominantly localized to the cell surface. The possibility exists that mAb 4B11 was directed to the 3'-nucleotidase, but the fact that the 3'-nucleotidase binds to Con A-Sepharose, and the mAb 4B11-reactive antigen did not, seems to suggest that *T. vivax* possesses two 35 kDa surface antigens, one of which is the 3'-nucleotidase. It will be important to purify these and other surface molecules to determine the ability of artificial or host antibodies to interfere with their target molecules, and thus parasite viability, despite the apparent immunological barrier of the trypanosomes' coat of variable antigens.

CHAPTER 7

Summary

African trypanosomes are the causative agents of an often chronic and debilitating disease of livestock and humans in a widespread region of sub-Saharan Africa. Although chemotherapeutic agents are available to combat both the human and animal diseases, they are expensive, often unobtainable and, in many areas, the parasites of livestock have developed resistance to these agents. Thus trypanosomiasis remains a formidable constraint on agricultural productivity and human health in Africa. The favoured solution to this problem would be a single-administration vaccine rather than the multiply administered prophylactic or therapeutic drugs. However, due to the successive expression of different variant surface glycoproteins (VSGs) during the course of infection, trypanosome populations are able to remain a step ahead of the host's immune responses. The repertoire of VSGs that could be expressed by a single trypanosome serodeme is too great for VSG to be of use as a vaccine. Therefore, the study of invariant membrane antigens is of interest for several reasons. Firstly, invariant molecules of the surface membrane, flagellar pocket membrane and endocytic pathway are potential targets for immunoprophylaxis Secondly, in laboratory studies these antigens could serve as specific markers for the purification of organelles. Finally, if characterized, these antigens could provide valuable information about the cell biology of trypanosomes. In this study, two novel invariant membrane proteins of *Trypanosoma vivax* have been identified and partially characterized using specific monoclonal antibodies.

A 35 kDa antigen was shown to be localized to the surface membrane of bloodstream forms of *T. vivax*, but some of the antigen appeared to be within the cell. Preliminary biochemical characterization of this antigen demonstrated that it was associated with both the membrane and soluble fractions of the parasite, with the majority of the molecule membrane-associated. The 35 kDa molecule had similar biochemical characteristics to the *T. vivax* 3'-nucleotidase, as it was of the same apparent molecular mass on SDS-PAGE gels and was partially membrane-associated and partially soluble. However, while the 3'-nucleotidase bound to Concanavalin A-Sepharose, the immunoreactive 35 kDa molecule did not. Therefore, it appears as though *T. vivax* has two surface proteins of 35 kDa, one of which has 3'-nucleotidase activity.

The main focus of this study was a 65 kDa antigen of *T. vivax*. This antigen is associated with tubulo-vesicular organelles which are involved in an intermediate part of the endocytic pathway. By comparison to mammalian cells, gp65 appears to be associated with endosome-like organelles of *T. vivax*. gp65 is an integral membrane glycoprotein with both N- and O-linked oligosaccharide side chains. In order to address the original objective of determining whether gp65 would be accessible to binding by antibody, protease protection and cross-linking studies were carried out. Results from these experiments suggest that gp65 exhibits properties of a transmembrane protein with both the trypsin cleavage sites and NH_2-cross-linking sites on the lumenal face of the organelles. The gp65 molecule appears to have cleavage sites for endoproteinase Glu-C and endoproteinase Asp-N on the cytoplasmic side of the membrane, however some of these sites seemed to be protected from cleavage by both enzymes. This suggests that the cytoplasmic portion of the gp65 may be bound to other molecules. The gp65 is able to form higher molecular weight complexes, possibly dimers and trimers by disulfide linkage. In addition, a 22 kDa molecule (p22), which is assumed to be a product of the 65 kDa molecule resulting through limited hydrolysis, is associated with gp65 by disulfide linkage. The 22 kDa portion of gp65 maintains the epitope which is recognized by the monoclonal antibody and since it is embedded in the membrane, probably contains a (the) membrane spanning region. In addition, p22 does not have the endoglycosidase H or O-glycosidase sensitive side chains detected on the entire gp65 molecule.

Recently, several lysosomal membrane glycoproteins (lgp) have been described in mammalian cells (reviewed by Fukuda, 1991). These molecules are transmembrane proteins with the bulk of the protein on the lumenal face of the lysosomes. The proteins have several conserved cysteine residues which are thought to separate the proteins into domains by intrachain disulfide linkages. Lgp's are heavily glycosylated containing both N- and O-linked glycans. The extent of glycosylation of these molecules is thought to be a mechanism of protection against the hydrolytic enzymes contained in the lysosomes. The lgp molecules are highly enriched in lysosomes, but a small percentage of them can be found on the plasma membrane. They have a short cytoplasmic tail, of which four residues have been implicated in the targeting of the lgp's to lysosomes. No function has been attributed to lgp molecules, in spite of the elucidation of entire DNA sequences for several of the proteins.

When comparing the *T. vivax* gp65 with well characterized proteins from mammalian cells, it is most similar to the lgp molecules described above. The putative cytoplasmic region of gp65 could function in targeting of the molecule to endosomes or serve as a marker for the particular organelle. As suggested by the protease protection results, the cytoplasmic portion of gp65 is protected from proteolysis in a percentage of the molecules. This could indicate that cytoplasmic proteins are bound to some of the gp65 molecules at the cytoplasmic domain. In mammalian cells and yeast, small *ras*-like GTP-binding proteins have been implicated in membrane fusion events. These proteins are associated with the cytoplasmic face of membranes in an organelle-specific manner, but it is likely that they bind to the cytoplasmic domain of a "resident" integral membrane protein of the specific organelle. By analogy, gp65 could serve as a marker of endosomes which binds specific cytoplasmic factors which in turn direct specific membrane fusion or fission events thus leading to the maturation of the endocytic pathway. In contrast, gp65 may bind molecules on the lumenal side of the endosomal membrane and act as a site of recognition for regulatory proteins, since the cytoplasmic domains of many proteins have been shown to be important in recognition and binding.

The potential for gp65 as a target for immunoprophylaxis has not been demonstrated fully in these studies, but should not be ruled out. gp65 resides in a relatively early endocytic compartment and is partially oriented on the lumenal face of these organelles, therefore, exogenous antibody could potentially bind to gp65 and interfere with the function of this protein. To test this, antibodies are needed that react with gp65 in its native form and which bind the portion of gp65 which is on the lumenal face of the membrane. Since mAb 4E1 did not bind to gp65 in its native state it could not be used in an experiment to determine its effect on parasite viability.

The function of gp65 is unknown and can only be speculated upon. Further work is necessary to characterize the protein and the organelles with which it is associated. Amino-terminal sequence analysis of gp65 was unsuccessful, thus no sequence data exists for this protein at this time. If gp65 has a fundamental role in the endocytic pathway of trypanosomes, it is expected to be relatively conserved and should also be present in *T. brucei* and *T. congolense*. However, the lysosomal cysteine protease of *T. congolense* is only found within this species (Mbawa et al., 1991). To approach this an expression library of *T. vivax* cDNA could be screened with the monoclonal antibody. DNA sequence data for the gp65 would permit the search for homologous proteins in other cells, including related species of trypanosomes. If transmembrane and cytoplasmic regions were predicted, possible signal sequences that might direct the targeting of the protein to endosomes could be sought within these regions. In addition, if a full length cDNA clone was obtained, it could be used to transfect mammalian cells in order to follow the distribution of the protein to determine whether it is localized in an endocytic organelle in these cells Such studies could provide better information in order to predict and possibly test the function of gp65 in *T. vivax*.

In conclusion, two novel invariant membrane antigens of *T. vivax* have been described. At least one of these molecules (gp65) has potential to be exploited as a target for immune intervention since it is oriented partly on the lumenal face of endocytic organelles which encounter exogenous material. The gp65 has served to better study the endocytic pathway of *T. vivax*, and if further characterized, could contribute to the better understanding of the cell biology of African trypanosomes, and may be of interest to cell biologists in general.

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