An Integral Membrane Glycoprotein Associated with an Endocytic Compartment of *Trypanosoma vivax*: Identification and Partial Characterization

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Abstract. A 65-kD glycoprotein (gp65) of *Trypanosoma (Duttonella) vivax* was identified using a murine monoclonal antibody (mAb 4E1) that had been raised against formalin-fixed, in vitro-propagated, uncoated forms. Intracellular localization studies utilizing the mAb in immunofluorescence on fixed, permeabilized *T. vivax* bloodstream forms and immunoelectron microscopy on thin sections of Lowicryl K_4M-embedded cells revealed labeling of vesicles and tubules in the posterior portion of the parasite. Some mAb-labeled vesicles contained endocytosed 10 nm BSA-gold after incubation of the parasites with the marker for 5–30 min at 37°C, and the greatest degree of colocalization was observed after 5 min. Double labeling experiments using the mAb and a polyclonal anti-variant surface glycoprotein (VSG) antibody to simultaneously localize both gp65 and 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 within the cell. Using the mAb for immunoblot analyses, gp65 was shown to be enriched in a fraction of solubilized membrane proteins eluted from either immobilized Con A or *Ricinus communis* agglutinin and was found to possess carbohydrate linkages cleaved by both endoglycosidase H and O-glycosidase, suggesting the presence of N- and O-linked glycans. Protease protection and crosslinking experiments suggest that gp65 is a transmembrane protein with trypsin cleavage and NH_2-crosslinking sites on the lumenal face of the vesicles.

AFRICAN trypanosomes are dependent for survival upon the uptake of nutrients from the blood of their mammalian host. Bloodstream forms of the parasite require transferrin and serum lipoproteins (Coppens et al., 1987; Black and Vandeweerd, 1989), and have been shown to acquire these nutrients by receptor-mediated endocytosis (Coppens et al., 1987). Recently, a low density lipoprotein receptor (Coppens et al., 1988, 1991) and a putative transferrin-binding protein (Schell et al., 1991) have been identified in *Trypanosoma brucei*. An extensive morphological description of the endocytic pathway of *T. brucei* and *T. congolense* has been made (Langreth and Balber, 1975; Coppens et al., 1987; Frevert and Reinwald, 1988; Webster and grab, 1988; Webster, 1989; Webster and Fish, 1989), but information concerning the mechanism of endocytosis in trypanosomes and specific markers of the pathway are lacking.

Recent studies of trypanosome cell biology have been directed toward the identification and purification of invariant cell surface or flagellar pocket molecules (Gbenle et al., 1986; McLaughlin, 1987; Coppens et al., 1988; Hide et al., 1989; Woods et al., 1989; Lee et al., 1990; Schell et al., 1991; Bringaud and Baltz, 1992; Ziegelbauer and Overath, 1992), and internal components of the endocytic pathway (Webster and Shapiro, 1990; Mbawa et al., 1991). These molecules may serve as potential targets for control of trypanosomiasis by immune intervention and provide the specific markers necessary for better understanding of the biochemistry and cell biology of the parasite. The surface of the bloodstream form parasite is completely covered by a coat of tightly packed variant surface glycoprotein (VSG)[1] molecules. Although this coat would appear to present an impenetrable barrier, masking underlying proteins (Cross, 1990), ligands such as transferrin are presumably able to bind to their receptors on *T. brucei*, which suggests that invariant molecules may be accessible. Since *T. vivax* expresses VSG molecules of lower molecular mass (Gardiner et al., 1987), and its surface coat appears to be less dense (Tetley and Vickerman, 1979) than that of the related species, *T. brucei* and *T. congolense*, invariant molecules of this parasite may be more accessible to external reagents, such as antibody.

1. *Abbreviations used in this paper*: DPBS, Dulbecco's PBS; DSP, dithio*bis*(succinimidyl propionate); DTSSP, 3,3′-dithio*bis*(sulfo-succinimidyl propionate); GUTat, Glasgow University Trypanozoon antigenic type; ILDat, International Laboratory for Research on Animal Diseases *Duttonella* antigen type; RCA, *Ricinus communis* agglutinin I; VSG, variant surface glycoprotein.

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T. vivax is an important parasite, causing disease in livestock of Africa and South America, and has a much wider geographical distribution than the more thoroughly studied *T. brucei* and *T. congolense* (reviewed in Gardiner, 1989). The aim of this study was to identify and characterize an invariant membrane protein of *T. vivax*, which may be accessible to antibody binding, as a step toward identifying potential target molecules for immunization. In this report we describe the raising of a monoclonal antibody to fixed, uncoated culture forms of *T. vivax* and its use in the identification and partial characterization of an invariant membrane glycoprotein associated with the endocytic pathway.

Materials and Methods

Materials

Reagents were obtained from the following sources: DE-52 from Whatman Laboratory Division (Maidstone, UK); hypoxanthine from BDH (Poole, UK); Eagle's MEM and RPMI-1640 from GIBCO (Paisley, Scotland); FBS from Northumbria Biologicals (Cramlington, UK); nonessential amino acids from ICN Flow (Irvine, UK); α-methyl mannoside, galactose, ovalbumin, proline, glutamine, hemin, complete Freund's adjuvant, incomplete Freund's adjuvant, trypsin (TPCK treated), Triton X-100, Triton X-114, and 3-aminopropyltriethylsilane from Sigma Chemical Co. (St. Louis, MO); Con A Sepharose from Pharmacia LKB (Uppsala, Sweden); *Ricinus communis* agglutin I–agarose from Vector Laboratories, Inc. (Burlingame, CA); endoglycosidase H, O-glycosidase, endoproteinase Glu-C, and endoproteinase Asp-N from Boehringer Mannheim (Mannheim, Germany); dithio*bis*(succinimidyl propionate) (DSP) and 3,3′-dithio*bis*(sulfo-succinimidyl propionate) (DTSSP) from Pierce Chemical Co. (Rockford, IL); cell culture flasks from Costar Corp. (Cambridge, MA); antipain, leupeptin, and E-64 from Cambridge Research Biochemicals (Northwich, UK); FITC-sheep anti-mouse Ig, ^{125}I-sheep anti-mouse Ig, mouse monoclonal antibody isotyping kit, Rainbow™ colored and Rainbow ^{14}C-methylated protein molecular weight markers from Amersham International (Amersham, UK); goat anti-mouse IgG conjugated to 5 nm gold from Biocell Research Laboratories (Cardiff, UK); SDS-PAGE reagents from Serva (Heidelberg, Germany); Citifluor from Agar Scientific (Stanstead, UK); Immobilon-P™ from Millipore Corp. (Bedford, MA); Centriprep-10 concentrators from Amicon (Beverly, MA); and Lowicryl K_4M resin from Chemische Werke Lowi (Waldkraiburg, Germany).

Parasites

Bloodstream forms. *T. (Duttonella) vivax* IL 1392, a rodent-infective stock, was derived from Y486, a stock originally isolated from a steer in Zaria, Nigeria (Leeflang et al., 1976). The derivation of International Laboratory for Research on Animal Diseases *Duttonella* antigen type (ILDat) 1.2, a clone of IL 1392 homogenous for variant antigen type, 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. brucei* Glasgow University Trypanozoon antigenic type (GUTat) 3.1 was derived from Treu 667 stock and cloned in Balb/c mice. 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). Parasites were grown in sublethally irradiated (600 rad) adult Swiss mice or Sprague-Dawley rats. Whole blood was collected just before the peak of parasitemia and diluted with an equal volume of phosphate-buffered (pH 8.0) saline glucose, supplemented with 0.1 mM hypoxanthine (Lonsdale-Eccles and Grab, 1987). Trypanosomes were separated from diluted blood by DE-52 chromatography (Lanham and Godfrey, 1970) using phosphate-buffered saline glucose/hypoxanthine for equilibration and elution.

Culture forms. The generation of 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. Blood stream 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 by Gumm (1991). On the third day, during the transformation of bloodstream forms to epimastigote forms, there exists a nondividing, noninfective form of the parasite that lacks an intact VSG surface coat. These are referred to as 3-d, uncoated, culture forms. Procyclic forms of *T. congolense* and *T. brucei* GuTat 3.1, which were established from the cloned bloodstream forms, were maintained in culture at 27°C in Eagle's MEM containing 25 mM Hepes (pH 7.4), Earle's salts, and 2 mM glutamine with the following additions: 10% (vol/vol) FBS, 1% (vol/vol) nonessential amino acids, 60 mM proline, 6.5 μg hemin/ml, and 200 μM hypoxanthine.

Monoclonal Antibodies (mAb 4E1)

3-d, uncoated, culture forms of *T. vivax* IL 1392 were fixed for 2 h in 4% (wt/vol) paraformaldehyde in 100 mM Pipes (pH 7.0). Fixed cells were washed in Dulbecco's PBS (pH 7.4) (DPBS) three times by centrifugation (750 *g*, 10 min) and resuspended in DPBS. Balb/c mice were injected with 2×10^7 fixed parasites emulsified in complete Freund's adjuvant for the primary immunization and incomplete Freund's adjuvant for subsequent boosts. Three injections were given at 21-d intervals. 4 d before the spleens were removed for fusion, the donor mice were given an intravenous injection of 2×10^7 fixed trypanosomes in DPBS. Fusion of mouse spleen cells with P3-X63-Ag8 mouse myeloma cells (Köhler and Milstein, 1975) was carried out according to the method described by Pearson et al. (1980). Tissue culture supernatants were screened for antibody on bloodstream forms of ILDat 1.2 by immunofluorescence and immunoblotting (see below). Hybridomas secreting antibodies of interest were double cloned by limiting dilution. On the basis of immunofluorescence screening and immunoblot analysis, mAb 4E1 (IgG_1 isotype) was selected for further investigation. The IgG fraction was isolated from ascites fluid by ammonium sulfate precipitation followed by DEAE-cellulose chromatography (Harlow and Lane, 1988).

Polyclonal Antisera to Variant Surface Glycoprotein (RxVSG)

Antisera were raised in rabbits against the 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., Inc., Del Mar, CA) for 18 h at 12 mA per cell in 50 mM NH_4HCO_3 (pH 8.2) containing 0.01% (wt/vol) SDS. Recovered protein was dialyzed against 4 liters of DPBS overnight at 4°C and then lyophilized. 100 μg of VSG was emulsified in complete Freund's adjuvant and injected subcutaneously at multiple sites along the backs of two rabbits. Three 50-μg injections of VSG emulsified in incomplete Freund's adjuvant were used to boost the rabbits at 28-d intervals. Antiserum was obtained from clotted blood after it was collected from the rabbits by cardiac puncture.

Immunofluorescence

Trypanosomes harvested from rats and purified by DE-52 chromatography were suspended in DPBS at 4×10^7 cells/ml and fixed for 1 h at room temperature by the addition of an equal volume of 4% (wt/vol) paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4). Cells were washed twice in DPBS by centrifugation at 750 *g* for 10 min and resuspended in the same buffer. Fixed cells were diluted in DPBS and 10^5 cells were spun onto slides coated with 2% (vol/vol) 3-aminopropyltriethylsilane in acetone using a Cytospin apparatus (Shandon Southern Products Ltd., Runcorn, UK) at 2,000 rpm for 10 min. Slides were incubated for 1 h in 1% (vol/vol) glutaraldehyde in 0.1 M phosphate buffer (pH 7.0) and washed for 10 min in DPBS containing 0.1% (wt/vol) BSA and 50 mM NH_4Cl (wash buffer). Cells were permeabilized for 2 h at room temperature using 0.1% (vol/vol) Triton X-100 in DPBS. Remaining free aldehyde groups were blocked by washing twice in wash buffer for a total of 30 min. 50 μl of mAb 4E1 (diluted 1:5,000 in wash buffer) was spread over the cells and incubated for 1 h at room temperature. Cells were washed for 30 min in wash buffer and then incubated for 1 h at room temperature in FITC-conjugated sheep anti-mouse Ig at a final dilution of 1:50. Cells were washed twice in wash buffer and then once in DPBS. Permanent mounts were made with Citifluor antifade mounting compound and examined using an Axiophot light microscope equipped with epifluorescent illumination and Differential Interference Contrast optics (Carl Zeiss, Oberkochen, Germany), and photographs were taken with Tmax 100 film from Kodak.

Immunoblotting

SDS-PAGE separation of trypanosome proteins was carried out as described

by Laemmli (1970) on 1.0-mm, 10–15% gradient gels with a 4% stacking gel unless otherwise stated. Proteins were electrophoretically transferred onto Immobilon-P^{TM} at 20 V for 18 h at 4°C, after partial renaturation by incubation of the gel in 50 mM Tris-HCl (pH 7.4) with 20% (vol/vol) glycerol for 1 h before transfer (Dunn, 1986). The blots were rinsed once in 10 mM Tris-HCl (pH 7.4) containing 150 mM NaCl and 0.1% (vol/vol) Tween 20 (TBST). Nonspecific protein binding sites were blocked by incubation of the blot in 5% (wt/vol) lowfat skim milk buffered with 10 mM Tris (pH 7.4) containing 15 mM NaCl for 1 h at room temperature. After blocking, the blots were rinsed twice with TBST, then incubated in mAb 4E1 diluted 1:5,000 in 5% (wt/vol) skim milk solution for 1–2 h at room temperature. Blots were washed with five changes of TBST over 30 min. The blots were incubated with 2 μCi of ^{125}I-anti-mouse Ig in 20 ml 5% (wt/vol) skim milk solution for 1 h, followed by extensive washing with TBST, then dried, and autoradiographs were produced by exposure to Fuji RX x-ray film with an intensifying screen at −70°C.

Membrane Preparation

Isolated bloodstream forms of *T. vivax* ILDat 2.1 were washed once in DPBS containing 0.1% (wt/vol) glucose and then suspended in ice-cold 50 mM Hepes (pH 7.4), 250 mM sucrose, and 25 mM KCl (SHK buffer) (Grab et al., 1987) containing 100 μg/ml of each of the protease inhibitors E-64, leupeptin, and antipain (Lonsdale-Eccles and Mpimbaza, 1986). Cells were lysed in an ice-cold French pressure cell at 2,500 psi and collected on ice. Unbroken cells and nuclei were removed by centrifugation at 750 *g* for 15 min, the postnuclear supernatant was centrifuged at 100,000 *g* in a Beckman 42.1 Ti rotor for 1 h at 4°C, and the pellet was retained.

Sodium carbonate treatment of membranes. Freshly prepared membranes were suspended in 10 mM Tris-HCl (pH 8.0), 150 mM NaCl (TBS) and then diluted 20-fold with ice-cold 0.2 M Na_2CO_3 (pH 11.5), essentially as described by Fujiki et al. (1982). Solubilized proteins were removed from membrane-associated proteins by centrifugation at 100,000 *g* at 4°C for 1 h. Supernatant and pellet fractions were examined by SDS-PAGE and immunoblotting.

Triton X-114 phase separation of membrane proteins. Freshly prepared membranes were solubilized in 2% (vol/vol) precondensed Triton X-114 in TBS (pH 7.4) on ice for 1 h with occasional mixing. Insoluble material was removed by centrifugation at 100,000 *g* at 4°C for 1 h. Phase separation of solubilized proteins was performed according to Bordier (1981).

Lectin precipitation. *T. vivax* ILDat 2.1 bloodstream forms were washed once in DPBS with 0.1% (wt/vol) glucose and lysed at a concentration of 5×10^8 cells/ml in 10 mM Tris-HCl (pH 7.4) containing 150 mM NaCl, 1 mM $MgCl_2$, 5 mM $CaCl_2$, 2% (vol/vol) NP-40, and 100 μg/ml of each protease inhibitor. The cells were frozen in liquid nitrogen and thawed at 37°C (two cycles) and insoluble material was then removed by centrifugation at 100,000 *g* in a 42.1 Ti rotor (Beckman Instruments, Inc., Fullerton, CA) for 1 h at 4°C. The detergent-soluble supernatant was mixed on a rocking table with one-tenth the volume of packed Con A–Sepharose beads or *Ricinus communis* agglutinin I (RCA)–agarose that had been preequilibrated with the lysis buffer for 1 h at room temperature. Unbound material was removed by centrifugation at 750 *g* for 5 min and the beads were washed at least five times with 10 vol 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-HCl (pH 7.4) containing 150 mM NaCl, 0.1% (vol/vol) NP-40, and 50 μg/ml protease inhibitors. The elution buffer was added to the beads in a 1:1 (vol/vol) ratio and incubated at 37°C for 15 min with occasional mixing. Eluted proteins were dialyzed at 4°C overnight against 4 liters of TBS (pH 7.4), concentrated using a Centriprep-10 concentrator, and then stored at −80°C.

Glycosidase digestions. Proteins eluted from RCA-agarose were precipitated by the chloroform/methanol/water method (Wessel and Flügge, 1984). 15 μg of total protein assayed according to Lowry et al. (1951) was used for each digestion. Protein precipitates were solubilized by boiling for 3 min in 10 μl of 0.25% (wt/vol) SDS, 0.1 M β-2-mercaptoethanol. No mercaptoethanol was added to the tubes in which O-glycosidase was to be added 4 mU of endoglycosidase H or 2.5 mU O-glycosidase, and in both cases, 0.5 μg of each of the protease inhibitors, were added in 20 μl of 50 mM sodium phosphate buffer (pH 5.8) and incubated at 37°C for 18 h. Digestions were stopped by adding an equal volume of 2× Laemmli sample buffer and boiling for 5 min. The digestion products were separated by SDS-PAGE, transferred to Immobilon, and then probed with mAb 4E1 as described above.

Nonreducing and two-dimensional SDS-PAGE gels. 25 μg of the proteins obtained from the detergent pellet of Triton X-114 was boiled for 5 min in Laemmli sample buffer without reducing agent, loaded into one well of a 1.0-mm-thick SDS-PAGE minigel, and electrophoresed at 20 mA constant current for 2.5 h. For two-dimensional gels, the lane was excised, equilibrated for 5 min in Laemmli sample buffer containing 50 mM DTT, drained, loaded horizontally onto the top of a second 1.5-mm-thick minigel with 100 μl Laemmli sample buffer (+DTT), and electrophoresed at 20 mA for 2.5 h. Gels were then blotted onto Immobilon as described above.

Protease protection experiments. Freshly prepared membranes were suspended gently in TBS (pH 8.0) using a loose-fitting Dounce homogenizer. 500-μl aliquots of membrane suspension (250 μg protein) were incubated at 37°C for 1 h with either trypsin (100 μg/ml), endoproteinase Asp-N (1.6 μg/ml), or endoproteinase Glu-C (40 μg/ml) in the presence or absence of 0.1% (vol/vol) Triton X-100. The reactions were stopped by adding 500 μl 2× Laemmli sample buffer and boiling for 5 min. 40-μl aliquots were used for SDS-PAGE/immunoblotting.

Cross-linking of proteins in a membrane fraction. 50 μl of a membrane suspension in TBS (pH 8.0) was diluted with 450 μl of 50 mM triethanolamine (pH 8.2), 100 mM NaCl, 1 mM EDTA, 5% (wt/vol) sucrose, and 5 μg of each of the three protease inhibitors. DSP (50 mM in DMSO) was added to the diluted membrane suspension to give a final concentration of 0.5 mM DSP and 1% (vol/vol) DMSO (Baskin and Ynag, 1982). For DTSSP treatment, 5 μl of a 100 mM stock in distilled water was added to 495 μl diluted membrane suspension to give a final DTSSP concentration of 1 mM. Membranes were incubated at room temperature for 2 h in the presence or absence of crosslinker, pelleted at 100,000 *g* for 45 min in a 70.1 Ti rotor (Beckman Instruments, Inc.), and boiled in Laemmli sample buffer with or without 50 mM DTT for 3 min. Samples were subjected to SDS-PAGE under reducing and nonreducing conditions, blotted, and probed with mAb 4E1 as described above.

Preparation of Trypanosomes for Electron Microscopy

Bloodstream forms of *T. vivax* ILDat 2.1 collected from DE-52 columns were washed once for 10 min in PSG/hypoxanthine (pH 7.4), pelleted by centrifugation at 750 *g* for 15 min, and then suspended in fixative containing varying glutaraldehyde concentrations (0.5–2%, vol/vol) with 4% (wt/vol) paraformaldehyde, 0.2% (wt/vol) picric acid, and 0.5 mM $CaCl_2$ in 0.1 M phosphate buffer (pH 7.4). After fixation for 1 h at room temperature, the trypanosomes were pelleted at 15,000 *g* for 3 min and the pellet was 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 progressive lowering of temperature (P.L.T.) method (Kellenberger et al., 1980; Armbruster et al., 1982). Pellets were polymerized at −30°C for 12 h by ultraviolet irradiation (350 nm peak emission).

Other samples of bloodstream form *T. vivax* ILDat 2.1 were fixed in 2% (vol/vol) glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). After 1 h of fixation, half of the sample was postfixed in 1% (wt/vol) osmium tetroxide and the other half was not. Both preparations were then stained *en bloc* overnight with 1% (wt/vol) aqueous uranyl acetate, and then dehydrated in graded acetone and embedded in Epon/Araldite resin with polymerization at 60°C. The Epon-embedded samples not postfixed in osmium were used for immunolocalization studies (see below), as were Lowicryl-embedded trypanosomes, to give a better indication of the structures being labeled by mAb 4E1.

Colloidal gold probes. Monodisperse gold sols of homogeneous sizes (5, 10, and 15 nm) were produced according to the method of Slot and Geuze (1985). The 15-nm gold sol was coupled to staphylococcal protein A, and the 5- and 10-nm gold sols were coupled to BSA (BSA-gold) using the method of Horisberger and Clerc (1985). BSA-gold conjugates were stored at 4°C in RPMI-1640 medium containing 0.02% (wt/vol) sodium azide, which was removed by extensive dialysis against RPMI-1640 containing 0.1 mM hypoxanthine before endocytosis experiments. A goat anti-mouse IgG 5-nm gold probe was used to localize mAb 4E1.

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 parafilm in a humidified chamber. Single labeling with mAb 4E1 was carried out as follows. Sections were preincubated by flotation of the grids on 3% (wt/vol) BSA in DPBS (pH 7.4) containing 0.5 mM $CaCl_2$ and 1 mM $MgCl_2$ for 30 min. Sections were then incubated with mAb 4E1 IgG (1:4,000) in DPBS/BSA for 1 h, washed three times (5 min each) in DPBS/BSA, and then labeled with the goat anti-mouse Ig 5-nm gold probe diluted 1:10 in DPBS/BSA for 1 h. Sections were then washed once in DPBS and twice in distilled water (5-min washes), stained for 10 min with 2% (wt/vol) aqueous osmium tetroxide, and stream washed with distilled water, followed by staining for 20 min with 2% (wt/vol) aqueous uranyl acetate.

Sections were counterstained with Reynold's lead citrate for 10 s, washed, dried, and examined in an EM10A electron microscope (Carl Zeiss) operating at 80 kV.

Double labeling with polyclonal rabbit anti-VSG antibody (RxVSG) and mAb 4E1 was performed as follows. After preincubation with DPBS/BSA, sections were incubated in RxVSG (diluted 1:1,000 in DPBS/BSA) for 1 h, washed three times (5 min each) in DPBS/BSA, labeled with 15 nm protein A–gold, and washed as above. This was followed by incubation for 30 min with a 1:100 dilution of normal rabbit serum to block any free protein A sites on the 15-nm probe. Sections were then washed again three times in DPBS/BSA and incubated with mAb 4E1, and labeling was performed according to the single antibody labeling procedure described above.

Endocytosis by Trypanosomes

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- and 10-nm BSA-gold was standardized to a solution absorbance of 1.0 at 525 nm, and 2×10^7 trypanosomes/ml were incubated at 37°C in suspensions containing probes either separately or mixed in equal volumes. Incubations were carried out for a maximum of 120 min, a period in which the trypanosomes maintained their motility. A 500-μl aliquot of cells taken after 15 s, and at various intervals thereafter, was added to 500 μl of double strength fixative (2% [vol/vol] glutaraldehyde, 8% [wt/vol] paraformaldehyde, 0.4% [wt/vol] picric acid, and 1 mM $CaCl_2$ in 0.2 M sodium phosphate buffer [pH 7.4]), and incubated for 1 h at room temperature. Cells were then processed into Lowicryl K_4M or epoxy resins and were used in single and double labeling experiments using mAb 4E1 and RxVSG as described above.

Results

Specificity of mAb 4E1

Immunofluorescence studies using permeabilized bloodstream forms of *T. vivax* showed that mAb 4E1 exhibits a punctate pattern of labeling (Fig. 1 *B*). For orientation, the same cells are shown with Nomarsky optics (Fig. 1 *A*), which demonstrates that the fluorescence label is localized in the posterior portion of the cell, between the flagellar pocket and the nucleus. Nonpermeabilized cells showed no fluorescence above background (not shown). On immunoblots, mAb 4E1 identified a 65-kD antigen (gp65) in lysates of bloodstream forms and uncoated culture forms of *T. vivax* (Fig. 2 *A*, lanes *1* and *2*), as well as epimastigotes (lane *3*) and metacyclics (lane *4*). The 55-kD band seen in lanes *1* and *2* was recognized by the second antibody alone (Fig. 2*A*, lane *5*), suggesting that it is parasite-associated mouse immunoglobulin acquired during growth in vivo. The 55-kD band was also detected in the uncoated culture forms (Fig. 2 *A*, lane *2*) as they are produced by seeding cultures with mouse-derived bloodstream forms and no subsequent passages are made. However, the 55-kD band was not detected in the other culture forms, epimastigotes (Fig. 2 *A*, lane *3*) and metacyclics (lane *4*), presumably because it is either degraded or diluted out with subsequent passages. MAb 4E1 recognized gp65 in *T. vivax*, but not in bloodstream or culture forms of two other species of African trypanosome, *T. brucei* and *T. congolense* (Fig. 2 *B*).

Membrane Association of gp65

The enrichment of gp65 in crude membranes of *T. vivax* (not shown) indicated its association with membranes, and the nature of this association was further studied by using different solubilization conditions. After extraction of a membrane fraction with sodium carbonate at alkaline pH, most of the gp65 was still membrane associated, as detected by immunoblotting (Fig. 3 *A*, lane *2*), suggesting a tight association of gp65 with the membrane. Further evidence came from the solubilization of membranes with Triton X-114 followed by phase separation (Fig. 3 *B*), where gp65 segregated into the detergent phase of Triton X-114 (Fig. 3 *B*, lane *3*). This illustrates the hydrophobic nature of gp65, suggesting that it is an integral membrane protein. In addition to the enrichment of gp65 by partition into the detergent phase of Triton X-114, a 22-kD band (p22) reactive with mAb 4E1 was highly enriched in this fraction.

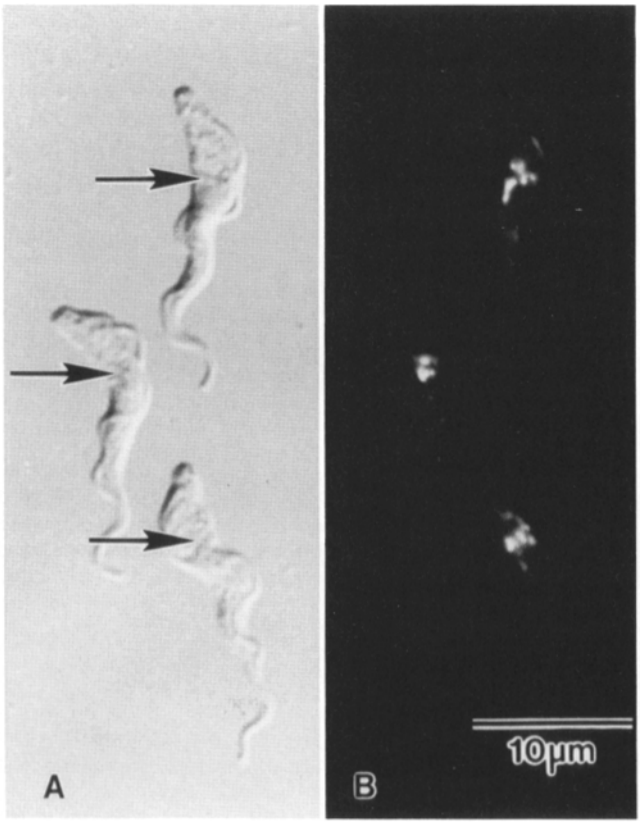

Figure 1. Immunofluorescence using mAb 4E1 on fixed, Triton X-100–permeabilized bloodstream form *T. vivax* ILDat 2.1 exhibits punctate labeling in the posterior portion of the parasite, indicated by arrows. (*A*) Nomarsky optics. (*B*) Immunofluorescence. Bar, 10 μm.

Oligomeric State of gp65

Proteins from the Triton X-114 detergent phase were electrophoresed under reducing conditions and compared, after immunoblotting, with a similar sample run under nonreducing conditions (Fig. 4 *A*). Under reducing conditions, both gp65 and p22 were detected (Fig. 4 *A*, lane *2*). Under nonreducing conditions, in addition to gp65, a higher molecular weight smear was observed in which there were two prominent bands, corresponding to approximate molecular masses of 130 and 190 kD (Fig. 4 *A*, lane *5*). These results suggest that gp65 is able to form higher molecular weight complexes, which are maintained by disulfide linkage. To investigate this possibility, two-dimensional SDS-PAGE gels were run (Fig. 4 *B*). Proteins from the Triton X-114 detergent pellet were first electrophoresed under nonreducing conditions (−DTT) similar to Fig. 4 *A* (lane *5*), and the lane was excised and then

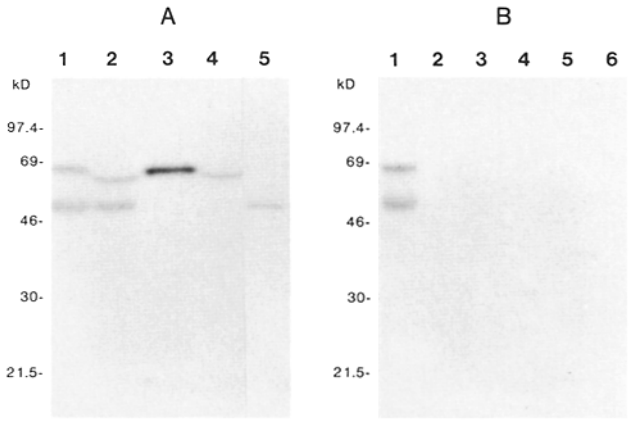

Figure 2. mAb 4E1 recognizes a 65-kD antigen in all life cycle stages of *T. vivax* specifically on immunoblots. Trypanosome lysates were separated by SDS-PAGE, electroblotted, and probed with mAb 4E1. (*A*) Life cycle stages of *T. vivax*: bloodstream forms (lane *1*), uncoated forms (lane *2*), epimastigote forms (lane *3*), and metacyclic forms (lane *4*) were probed with mAb 4E1. In lane *5* is a sample of bloodstream forms that has been probed with the second antibody, ^{125}I-anti-mouse alone to demonstrate that the 55-kD band seen in the lysates is parasite-associated mouse Ig. (*B*) Immunoblot to test the species specificity of mAb 4E1 reactivity. *T. vivax* bloodstream forms (mouse-derived), lane *1*; *T. brucei* bloodstream forms (rat-derived), lane *2*; *T. brucei* procyclic forms, lane *3*; *T. congolense* bloodstream forms (rat-derived), lane *4*; *T. congolense* procyclic forms, lane *5*; *T. congolense* epimastigote forms, lane *6*. 25 μg total protein was loaded in each lane.

electrophoresed in a second dimension under reducing conditions (+DTT). The high molecular weight complexes seen in the one-dimensional nonreduced sample are resolved to gp65 and p22, where p22 is aligned below gp65 in the second dimension on the immunoblot.

Effect of Glycosidases on the Apparent Molecular Mass of gp65

Enrichment of gp65 by binding to Con A–Sepharose (Fig. 5, lane *1*) or RCA-agarose (Fig. 5, lanes *2–5*) and elution of

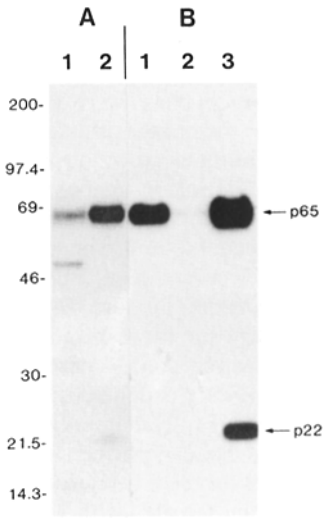

Figure 3. Membrane association of gp65 and enrichment of another immunoreactive protein of 22 kD (p22). Crude membranes from *T. vivax* ILDat 2.1 bloodstream forms were prepared and extracted with 0.2 M Na_2CO_3, pH 11.5 (*A*) or solubilized with 2% Triton X-114 and subjected to phase separation (*B*). Samples were run on SDS-PAGE, blotted, and probed with mAb 4E1. (*A*) Lane *1*, Na_2CO_3 supernatant; lane *2*, Na_2CO_3 pellet. (*B*) Lane *1*, Triton X-114 solubilized membranes; lane *2*, Triton X-114 aqueous phase; lane *3*, Triton X-114 detergent pellet. 25 μg total protein was loaded in each lane.

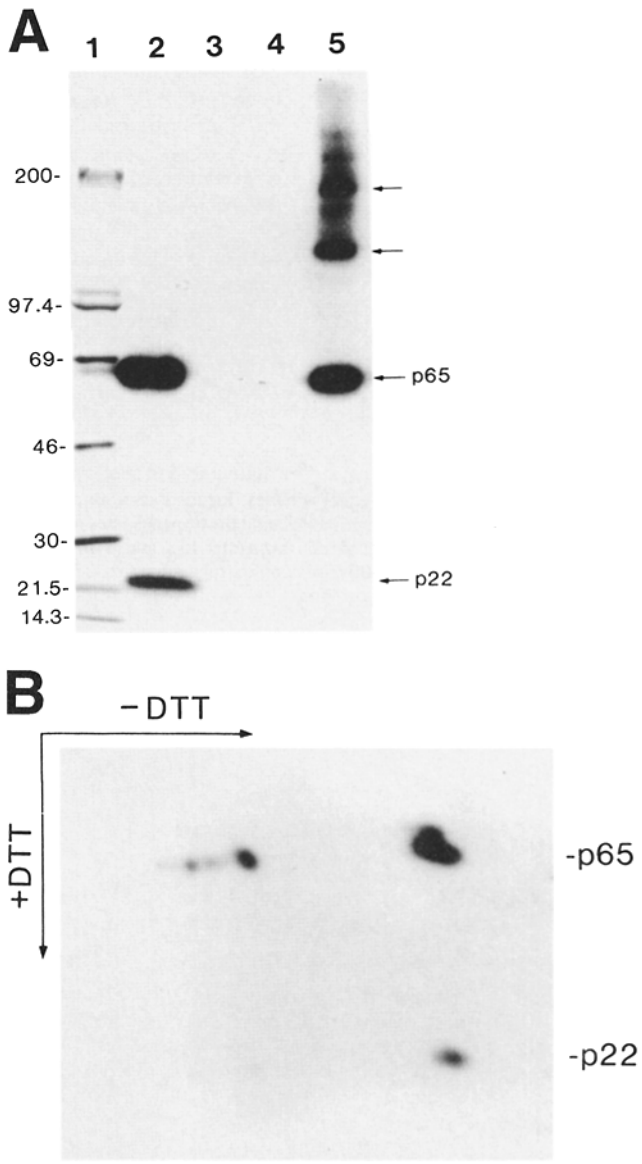

Figure 4. Oligomeric state of gp65. (*A*) Proteins from the detergent phase of Triton X-114 phase separation were electrophoresed under reducing conditions (lane *2*) and the pattern of immunoreactive proteins was compared with the proteins run under nonreducing conditions (lane *5*) after immunoblotting. Lanes *3* and *4* contain Laemmli sample buffer without DTT. Lane *5* demonstrates that in the nonreduced sample p22 is not present, but there are higher molecular weight species; two prominent bands are indicated by arrows. (*B*) Two-dimensional gel of Triton X-114 detergent pellet proteins, blotted and probed with mAb 4E1. Proteins were separated under nonreducing conditions in the first dimension (4% stacking gel, 7.5% separating gel) and reducing conditions in the second dimension (4% stacking gel, 10–15% separating gel). p22 is aligned below the gp65 molecule in the second dimension. The high molecular weight complexes are resolved to gp65 and p22. 25 μg total protein loaded in each lane.

the proteins with the specific competing sugars suggested that gp65 has N-linked glycans containing both mannose and galactose residues (Fig. 5). To determine the nature of the presumptive oligosaccharide linkages of gp65, proteins eluted from RCA-agarose were treated with endoglycosidase H or O-glycosidase. Treatment with either glycosidase

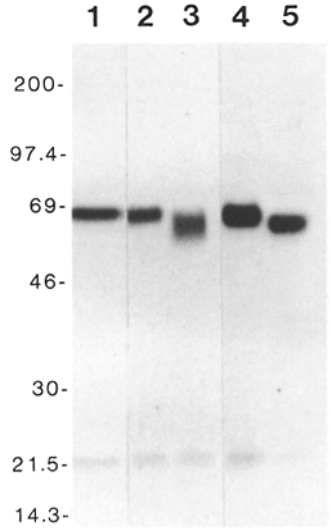

Figure 5. gp65 is a glycoprotein with N- and O-linked glycans. NP-40 solubilized membranes were incubated with the immobilized lectins, Con A or RCA. Bound proteins were recovered by elution with the specific competing sugars: 0.5 M α-methyl mannoside for Con A (lane *1*) and 0.5 M galactose for RCA (lanes *2–5*). Proteins eluted from RCA were digested with either 4 mU endo H (lane *3*) or 2.5 mU O-glycosidase (lane *5*), separated on SDS-PAGE under reducing conditions, blotted, and probed with mAb 4E1. Lanes *2* and *4* are mock-treated controls for glycosidase digests. 25 μg total protein was loaded in each lane.

resulted in a reduction in the apparent molecular mass of gp65 but had no effect on the relative mobility of p22 (Fig. 5, lanes *3* and *5*). These results suggest that gp65 is a glycoprotein with both N-linked and O-linked glycans, but that the p22 portion does not contain any oligosaccharide linkages. In addition, these results suggest that the mAb 4E1 is reactive with a protein epitope as opposed to a carbohydrate epitope, since the mAb still recognizes the protein after glycosidase digestion.

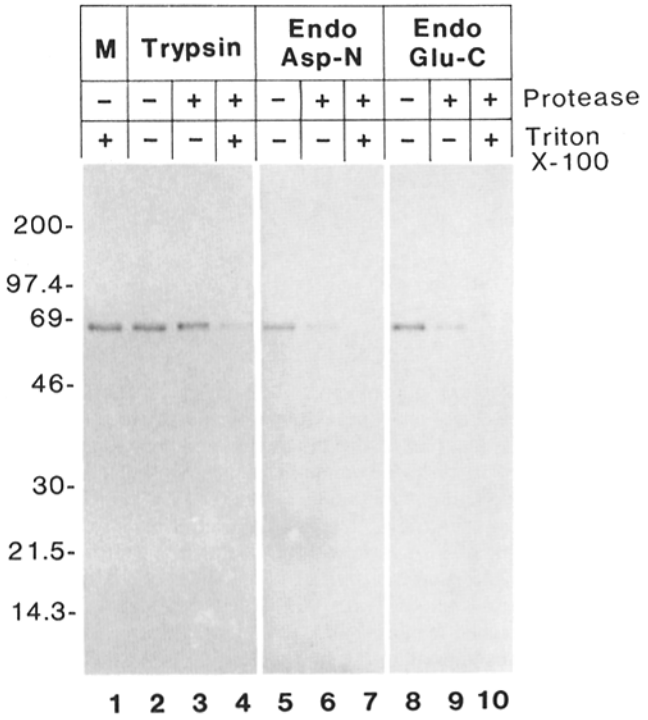

Figure 6. Membrane orientation of gp65. Membranes were treated with trypsin, endoproteinase Asp-N, or endoproteinase Glu-C in the presence or absence of 0.1% Triton X-100. Samples were run on SDS-PAGE, blotted, and probed with mAb 4E1. Lane *1*, membranes (*M*) treated with 0.1% Triton X-100; lanes *2*, *5*, and *8*, mock-treated controls for endogenous protease activity; lanes *3*, *6*, and *9*, membranes treated with trypsin (100 μg/ml), endo Asp-N (1.6 μg/ml), and endo Glu-C (40 μg/ml), respectively; lanes *4*, *7*, and *10*, 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.

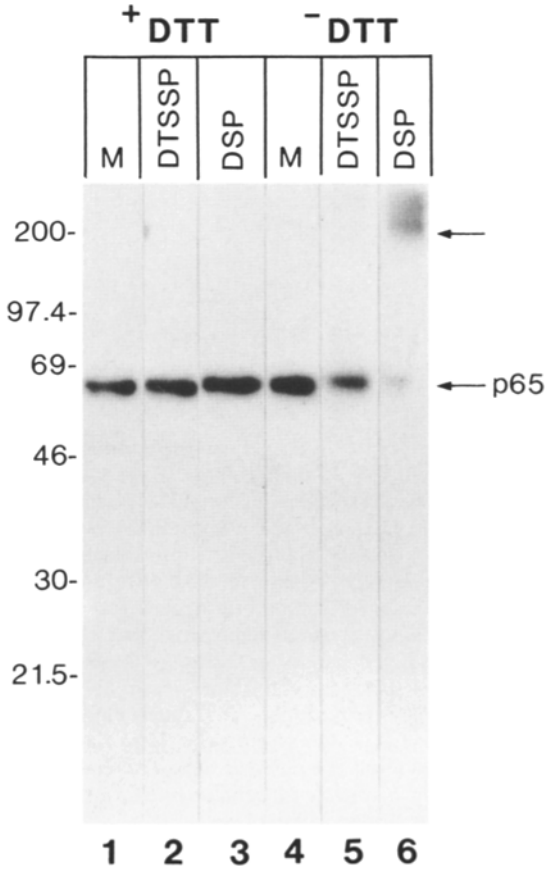

Figure 7. Chemical cross-linking of gp65 in membranes. Membranes (250 μg protein) were either mock-treated (lanes *1* and *4*), or treated with the membrane-impermeable crosslinker, DTSSP (lanes *2* and *5*) or the membrane-permeable crosslinker, DSP (lanes *3* and *6*). 30 μg total membrane protein was electrophoresed under reducing conditions (+DTT, lanes *1–3*) or nonreducing 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 crosslinker DSP. 30 μg protein was loaded in each lane.

Membrane Orientation of gp65

The first approach to investigating the membrane orientation of gp65 was to determine its accessibility to exogenous proteases. Aliquots of a membrane fraction were treated with various proteases in the presence and absence of Triton X-100, and gp65 was detected by immunoblotting. Treatment of membranes with trypsin had no effect on the gp65 unless Triton X-100 was present in the incubation mixture (Fig. 6, lane *4*). This suggests that the cleavage site for trypsin is on the lumenal face of the vesicles. However, gp65 is partially cleaved by both endoglycosidase Asp-N and endoglycosidase Glu-C in the absence of detergent, and is fully cleaved when Triton X-100 is present (Fig. 6, lanes *6* and *7*, respectively), indicating that there are cleavage sites for these two enzymes on both sides of the vesicles. Membranes treated with Triton

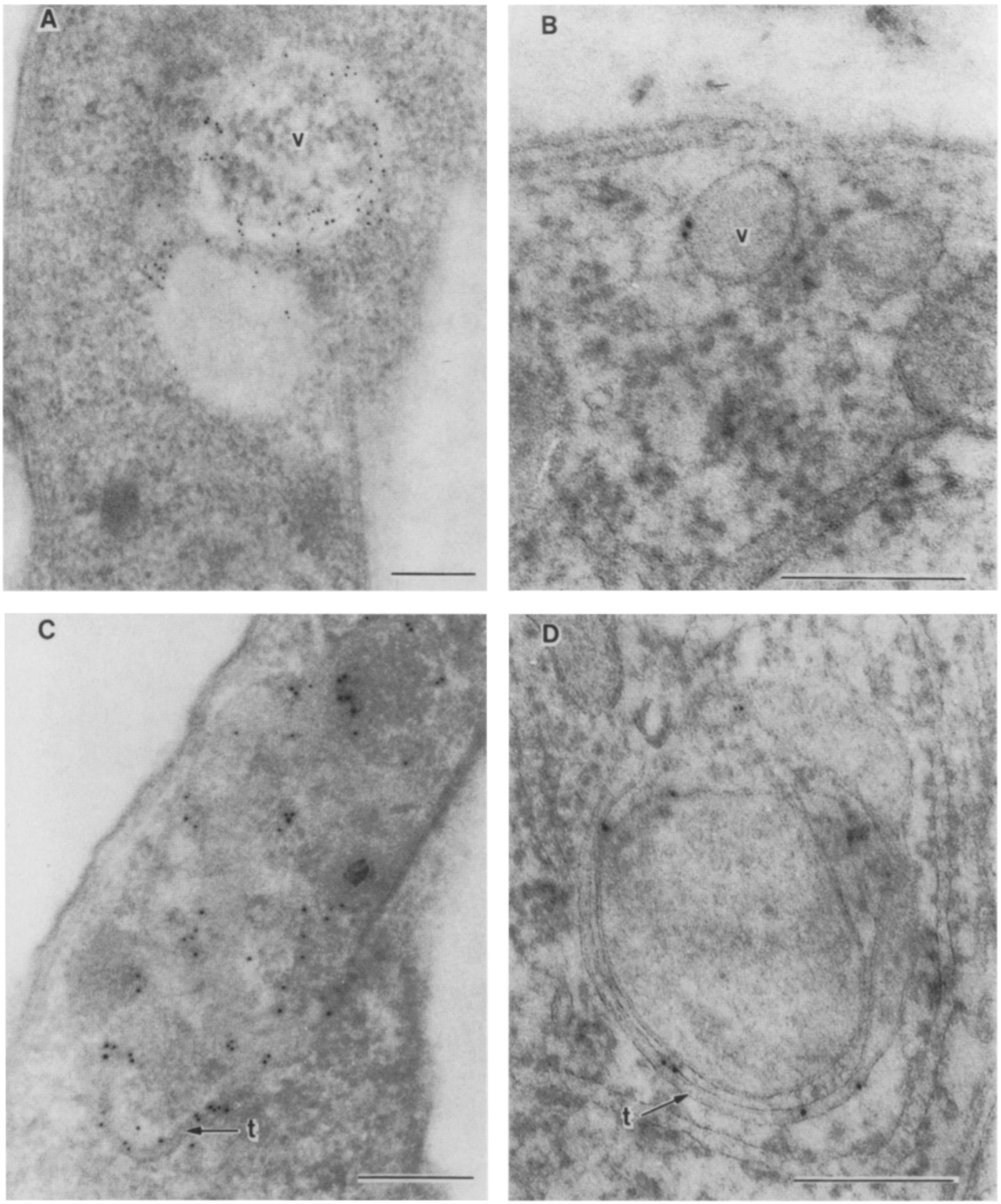

Figure 8. Immunolocalization of gp65 to the membrane of vesicles and tubular profiles using mAb 4E1 to label ultrathin sections of Lowicryl-embedded (*A* and *C*) and Epon-embedded (*B* and *D*) bloodstream forms of *T. vivax* ILDat 2.1. Vesicular (*v*) and tubular profile (*t*) labeling seen in the Lowicryl-embedded sections (*A* and *C*) is more clearly localized to the membrane in Epon sections (*B* and *D*, respectively). Bar, 0.2 μm.

X-100 (Fig. 6, lane *1*) or incubation of membranes in the absence of proteolytic enzymes (Fig. 6, lanes *2*, *5*, and *8*) demonstrate that gp65 is not degraded under control conditions.

Crosslinking experiments using membrane-permeable (DSP) and impermeable (DTSSP) crosslinkers show that only DSP crosslinks gp65 efficiently (Fig. 7, lane *6*). When crosslinked membranes were electrophoresed under reducing conditions (Fig. 7, lanes *2* and *3*), as a control for the reversibility of the crosslinking, gp65 was detected after both DSP and DTSSP treatments. Untreated membranes were also run under reducing and nonreducing conditions (Fig. 7, lanes *1* and *4*) to ensure that the gp65 was not crosslinked

before treatment. These results show that gp65 is inaccessible to crosslinking unless the reagent can traverse the membrane. In both of these experiments, the majority of the vesicles seemed to be intact, as suggested by the results from the trypsin digestions (above). If the partial degradation of gp65 with endo Asp-N and endo Glu-C seen in the absence of detergent was due to a proportion of vesicles being inside out, then partial degradation of gp65 with trypsin would be expected in the absence of detergent. In addition, the membrane-impermeable crosslinker DTSSP would have been able to crosslink more of the gp65. Taken together, these results provide evidence that gp65 is a transmembrane protein with the trypsin cleavage and crosslinking site(s) on the lumenal face of the vesicles.

Intracellular Localization of gp65

A more detailed localization of gp65 was obtained using mAb 4E1 for immunolabeling of thin sections of glutaraldehyde-fixed bloodstream form trypanosomes embedded in either Lowicryl K_4M or Epon resin. Fig. 8 demonstrates that the mAb 4E1 label is specifically associated with cisternal and vesicular structures. Ultrathin sections of Lowicryl- (Fig. 8, *A* and *C*) and Epon- (Fig. 8, *B* and *D*) embedded cells were used for immunolabeling. Although the degree of labeling achieved on Lowicryl K_4M sections was much greater, membrane preservation and visualization was better in epoxy sections. Therefore, epoxy-embedded trypanosomes are shown only to illustrate more clearly the membrane association of the label and the vesicular (Fig. 8, *A* and *B*) and the cisternal or tubular (Fig. 8, *C* and *D*) nature of these structures. No labeling was observed on the surface membrane, flagellar pocket, coated vesicles, Golgi apparatus, endoplasmic reticulum, or nucleus. Furthermore, no labeling was observed in control experiments using either normal mouse serum or the second antibody gold probe alone (not shown).

Endocytosis of BSA-Gold

Results from the immunolocalization studies demonstrate that gp65 localizes to vesicular and cisternal membranes in the posterior portion of bloodstream form parasites. Since the organelles involved in endocytosis are located exclusively in 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-labeled 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 more appropriate conditions. Initial experiments examined uptake of 5- 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- and 10-nm BSA-gold probes are rapidly endocytosed and retained in similar organelles (not shown) and during the incubation time, up to 120 min, cells remained viable, as judged by motility. Furthermore, when

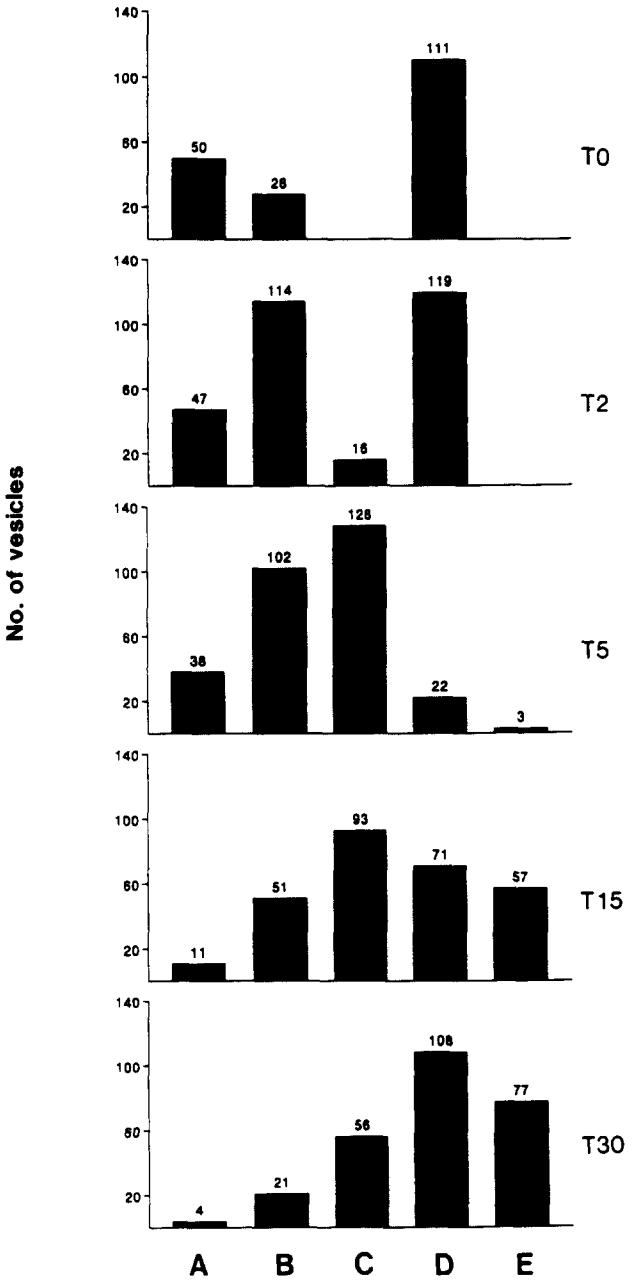

Figure 9. Graphic representation of the association of mAb 4E1 labeling and endocytosed BSA-gold with certain vesicle populations with time. 50 trypanosome sections showing both flagellar pocket and nucleus, and selected only if there was visible endocytosed BSA-gold, were counted. The flagellar pocket (*A*) and four vesicle types (*B–E*) were counted in each section and scored for the presence or absence of endocytosed BSA-gold and the mAb 4E1 label. The vesicle types considered were small vesicles in the proximity of the flagellar pocket containing <30 particles of endocytosed BSA-gold (*B*); vesicles showing colocalization of endocytosed BSA-gold and mAb 4E1 (*C*); vesicles showing only mAb 4E1 labeling (*D*), and large vesicles in the proximity of the nucleus containing >30 particles of endocytosed BSA-gold (*E*). Graphs T0–T30 represent the scored vesicles in each category at different intervals after initiation of endocytosis of BSA-gold.

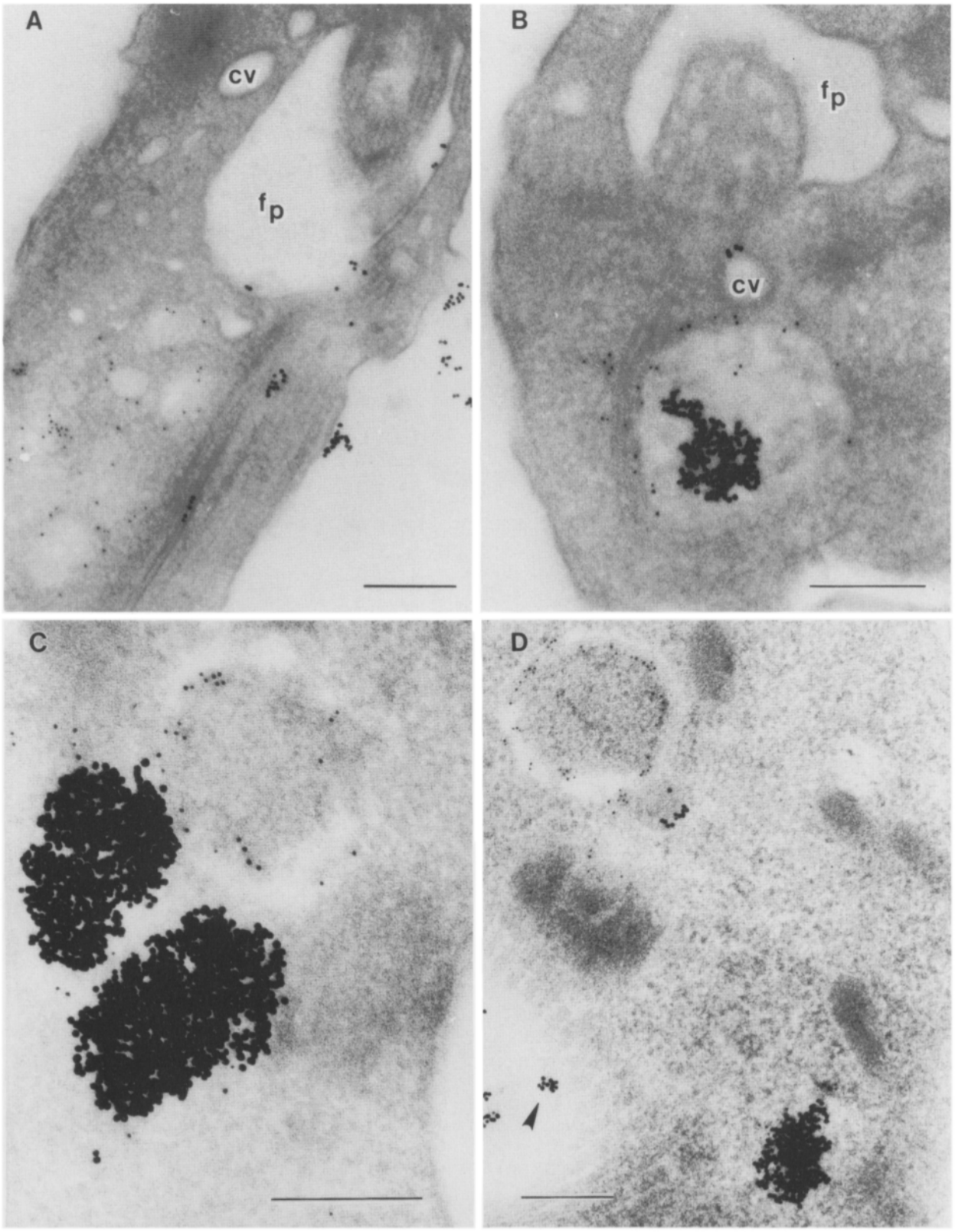

Figure 10. Colocalization of mAb 4E1 with endocytosed BSA-gold. *T. vivax* bloodstream forms were incubated in medium containing endocytosed BSA-gold (10 nm probe) for 15 s (*A*), 5 min (*B*), 15 min (*C*), and 30 min (*D*) before fixation and Lowicryl embedding. Ultrathin sections were labeled with mAb 4E1 followed by goat anti-mouse IgG (5-nm probe). Endocytosed BSA-gold shows the greatest colocalization with the mAb 4E1 label on sections of cells from the 5-min time point (*B*). Less colocalization of the two markers is seen at the 15- (*C*) and 30-min (*D*) time points. *fp*; flagellar pocket; *cv*; coated vesicles; arrowhead in *D* indicates extracellular BSA-gold. Bar, 0.2 μm.

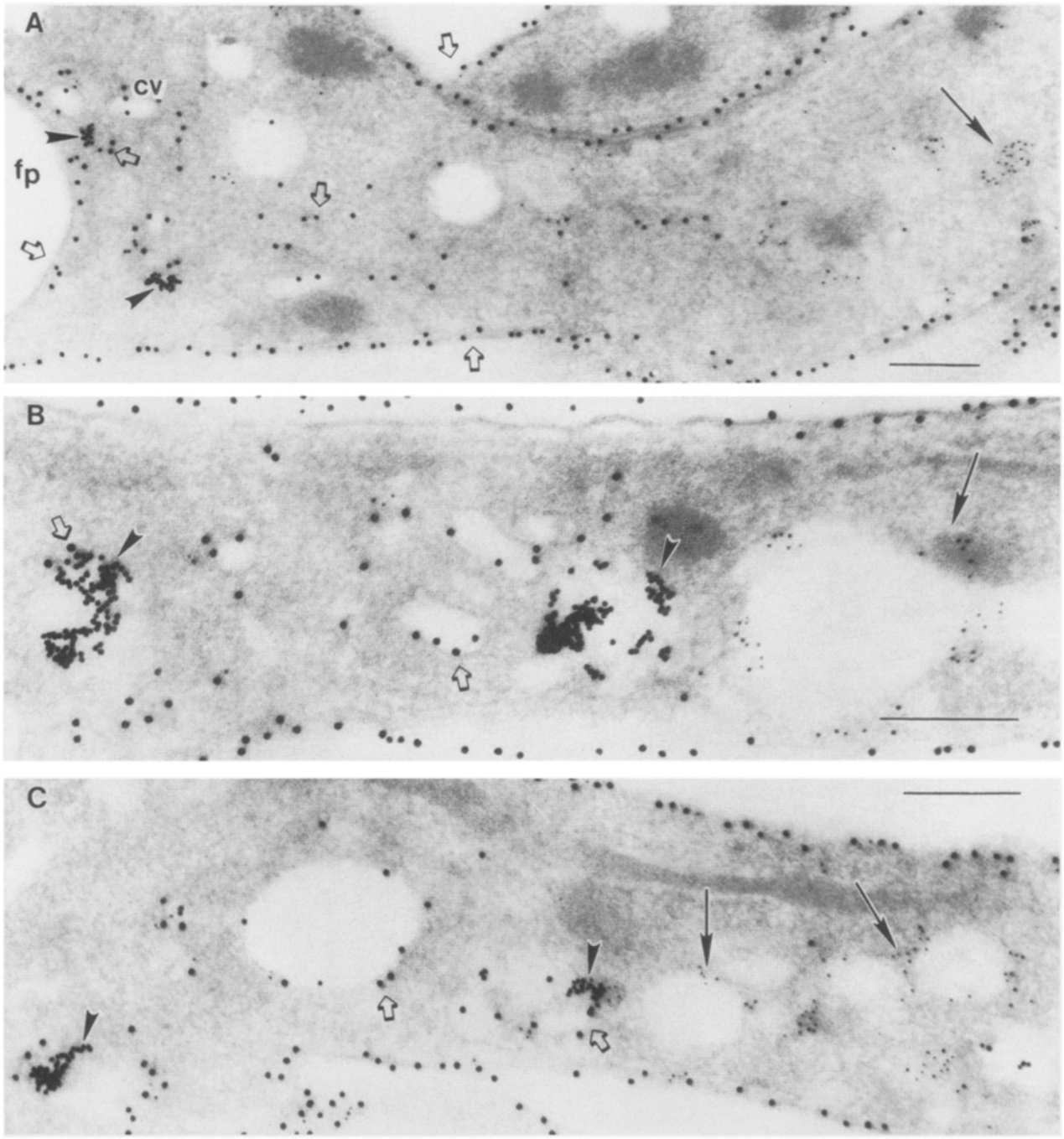

Figure 11. Double antibody labeling using mAb 4E1 and RxVSG on *T. vivax* bloodstream forms after incubation with BSA-gold. *T. vivax* incubated in medium containing BSA-gold (10-nm probe) for 1 min (*A*), 2 min (*B*), 3 min (*C*), 4 min (*D*), 5 min (*E*), and 15 min (*F*) before fixation and Lowicryl embedding. Ultrathin sections were labeled with RxVSG and then protein A–gold (15-nm probe) followed by mAb 4E1 and goat anti–mouse Ig (5-nm probe) as outlined in Materials and Methods. The RxVSG probe (15 nm), indicated by a hollow arrow, labeled the cell surface, flagellar pocket (*fp*), and coated vesicles (*cv*), as seen in *A*; tubular profiles (*t*) in *D*; and the Golgi apparatus (*g*) in *F*. The position of endocytosed BSA-gold (10 nm) is indicated with an arrowhead and mAb 4E1 label (5 nm) is indicated with a solid arrow. Bar, 0.2 μm.

combined in a single incubation, the two probes were always colocalized. Thus, for better visualization, all further endocytosis experiments were performed using 10 nm BSA-gold.

In endocytosis experiments BSA-gold particles were present in the flagellar pocket and coated vesicles after 15 s, which was the minimum time needed to mix the trypanosomes with the BSA-gold suspension and remove an aliquot for fixation (Fig. 9). Incubation of cells in BSA-gold for 5–30 min resulted in the accumulation of endocytosed marker within many internal cisternal 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

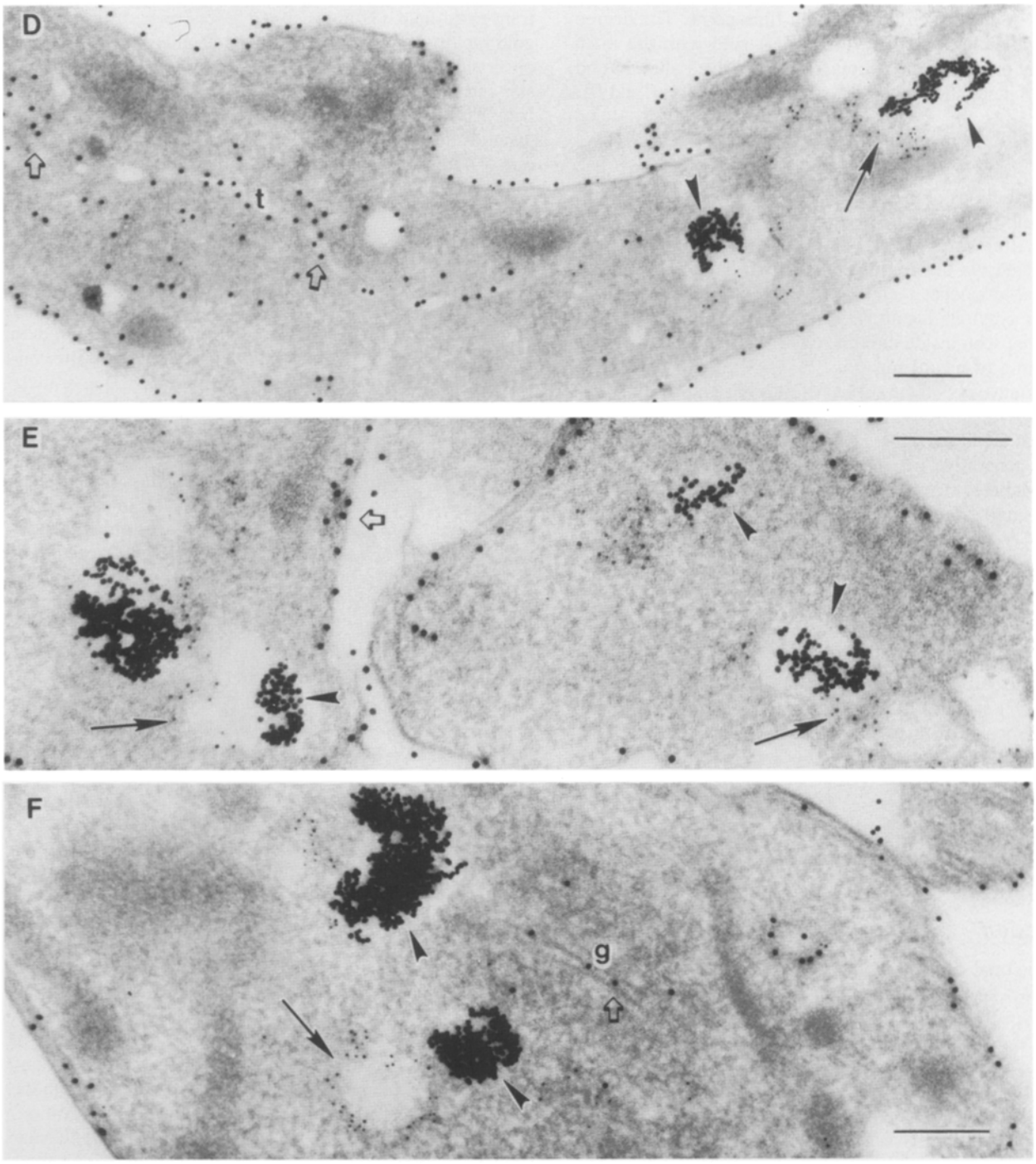

Figure 11.

in the flagellar pocket and coated vesicles at the 15- and 30-min time points (Fig. 9). After 30 min the BSA-gold marker appeared to be mostly aggregated within vesicles that are in close proximity to the nucleus and that are perhaps lysosomes. No further movement of endocytosed BSA-gold could be discerned at time points up to 2 h (not shown).

Colocalization of mAb 4E1 with Endocytosed BSA-Gold in T. vivax

Since endocytosed BSA-gold occupied different organelles with increasing incubation times, from 15 s to 30 min, these time points were used for the colocalization of BSA-gold and mAb 4E1. Immunolocalization with mAb 4E1 shows that, after incubation of trypanosomes in BSA-gold for 15 s (Figs. 9 and 10 *A*), the membrane-bound compartments labeled with the antibody do not contain the endocytosed marker. However, varying degrees of colocalization of the mAb 4E1 with vesicles containing endocytosed BSA-gold are seen at the subsequent time points (Fig. 9 and 10, *B–D*). The endocytosed marker and the mAb-labeled vesicles show the greatest degree of colocalization after incubation of the parasites in BSA-gold for 5 min (Fig. 9 and 10 *B*). However, not all vesicles that labeled with mAb 4E1 contained endocy-

tosed BSA-gold, even at the 5-min time point. The degree of colocalization of endocytosed BSA-gold with the mAb 4E1 label progressively decreases in cells fixed after 15 and 30 min of incubation with BSA-gold (Fig. 9 and 11, *C* and *D*).

Immunolocalization of mAb 4E1 and RxVSG on T. vivax *Containing Endocytosed BSA-Gold*

The distributions of mAb 4E1 and RxVSG labels were compared with those of endocytosed BSA-gold at different time points. After 1 min, both VSG and BSA-gold appear to be internalized via the coated vesicles that have budded from the flagellar pocket membrane (Fig. 11 *A*). In agreement with our previous results, BSA-gold is rapidly internalized and can be seen inside the cell, close to the flagellar pocket, after 1 min of incubation (Fig. 11 *A*). RxVSG label is also found extensively on the surface of the cells and within many vesicles (Fig. 11, *A–D*) and tubular profiles (Fig. 11 *D*), as well as in the Golgi (Fig. 11 *F*). Vesicles labeled with mAb 4E1 are generally further from the flagellar pocket than the RxVSG-labeled structures; thus, the two labels only colocalize to a small extent. At 1, 2, and 3 min, some of the endocytosed BSA-gold colocalizes with the RxVSG label (Fig. 11, *A–C*), although most of the two labels are found in distinct structures. By 2–3 min (Fig. 11 *C*), some of the endocytosed BSA-gold is colocalized with the mAb 4E1 and by 4 and 5 min (Fig. 11, *D* and *E*) this association is more pronounced. After 5 min, very little BSA-gold is observed within the region of the cell predominantly labeled by RxVSG. At 15 min (Fig. 11 *F*) some of the BSA-gold is colocalized with mAb 4E1, but most is distinct, as observed previously (Fig. 10 *D*). A low level of cell surface labeling with mAb 4E1 is visible on some sections (Fig. 11, *A* and *C*). Control experiments revealed this to be an artifact due to incomplete blocking of protein A on the 15-nm probe. No other cell surface labeling occurred.

Discussion

A monoclonal antibody, 4E1, isolated while screening for those reactive with *T. vivax* invariant antigens, identified a species-specific, internal membrane glycoprotein, termed gp65. Using this antibody in immunofluorescence studies, gp65 was localized to structures in the posterior portion of permeabilized cells and immunoelectron microscopy clearly showed that the majority of gp65 is associated with the membranes of vesicular or cisternal organelles. In previous morphological studies of the related trypanosomes *T. brucei* and *T. congolense*, the endocytic organelles were described as a tubulo-vesicular network found exclusively in the posterior portion of the cell (Frevert and Reinwald, 1988; Webster and Grab, 1988; Webster, 1989; Webster and Fish, 1989). To demonstrate a possible involvement of the gp65-associated organelles in the endocytic pathway of *T. vivax*, colocalization studies using mAb 4E1 and BSA-gold were performed. The results of initial endocytosis experiments with *T. vivax* showed that with increasing time of incubation in medium containing BSA-gold, the marker occupied different areas within the cell. Specifically, it moved from the flagellar pocket and coated vesicles, where it is found within 15 s, to spherical, "lysosome-like" organelles in close proximity to the nucleus after 30 min of incubation. These results are in agreement with previous studies that followed endocytosed transferrin-gold (Tf-gold) in *T. brucei*, where transferrin-gold rapidly entered the cell via the flagellar pocket and was observed in lysosome-like organelles after 10–30 min (Webster and Grab, 1988). In addition, Mbawa et al. (1991) localized a lysosomal cysteine protease in *T. congolense* to organelles that accumulated endocytosed BSA-gold within 30 min of addition of the labeled BSA. Using time points of 15 s to 30 min for colocalization studies, it was shown that the greatest degree of colocalization of mAb 4E1 and BSA-gold occurred after 5 min of endocytosis, but decreased at the later time intervals. Indeed, there was little association of mAb 4E1 labeling with the lysosome like organelles in which the endocytosed BSA-gold appeared to accumulate after 15–30 min. Similarly, no antibody labeling occurred in structures containing BSA-gold immediately (15 s) after initiation of endocytosis of the exogenous label. Together these observations suggest that gp65 is located in an intermediate part of the endocytic pathway of *T. vivax*. The term intermediate is used to distinguish the mAb 4E1–labeled organelles from the early endocytic organelles (the flagellar pocket and coated vesicles) and the organelles in which BSA-gold is ultimately concentrated, which are probably lysosomes. Therefore, based on the tubulo-vesicular structure of the gp65-associated organelles and the fact that they contain endocytosed material after relatively short incubation times, it is possible that these *T. vivax* organelles are similar to the endosomes described for mammalian cells (Helenius et al., 1983). Whether they would be more similar to early or late endosomes (Schmid et al., 1988) is not known, but with the availability of gp65 as a marker of this endocytic compartment in *T. vivax*, more detailed studies can be initiated to improve characterization of the organelles.

The membrane association of gp65, seen in the immunolocalization studies, is supported by biochemical data. The gp65 is resistant to extraction from the membrane by sodium carbonate treatment and partitions into the detergent phase of Triton X-114, thus exhibiting properties characteristic of integral membrane proteins. Results from nonreducing and two-dimensional (nonreduced/reduced) gels suggest that gp65 can exist as an oligomer, possibly as dimers and trimers linked via disulfide linkage. The immunoreactive p22 molecule is most simply interpreted as being a proteolytic product of the gp65, which results from incomplete proteolysis of gp65 and which contains the mAb 4E1 epitope. After cleavage it appears that this fragment remains associated with the rest of the gp65 molecule by disulfide linkage. In addition, some gp65 molecules appear to form higher molecular weight complexes that may be dimers or trimers, but most of the gp65 appears to be a monomer.

Binding of gp65 to both Con A and RCA indicates the presence of mannose and galactose residues, and since carbohydrate linkages are cleaved by endoglycosidase H and O-glycosidase, both N- and O-linked glycans are present. Although the p22 moiety is enriched after lectin binding, it does not appear to contain any oligosaccharide linkages, since it is unaffected by glycosidases. This suggests that p22 is coenriched with gp65 due to its physical association by disulfide linkage. Recently, a component ("plgp57") of a prelysosomal compartment of Madin-Darby bovine kidney cells has been characterized (Park et al., 1991) that has biochemical characteristics similar to those of a family of lysosome-associated membrane glycoproteins, *lamp* molecules (reviewed in Fukuda, 1991). These molecules are heav-

ily glycosylated, integral membrane proteins of unknown function. However, since glycosylation, especially O-glycosylation, can confer resistance to proteolysis (reviewed in Jentoft, 1990), it has been speculated that the *lamp* proteins, some of which possess O-linked oligosaccharides, protect the lysosomal membrane from hydrolase damage (Fukuda, 1991). The function of gp65 is unknown, but it appears to posses some of the characteristics of plgp57 and *lamp* molecules, in that it is an integral membrane glycoprotein with O-linked glycans and is located in endocytic organelles. In addition, membrane orientation studies showed that gp65 is a transmembrane protein with trypsin cleavage and DSP crosslinking sites on the lumenal face of the vesicles. Low amounts of *lamp* molecules have been shown to be present in the surface of some mammalian cells (Lippincott-Schwartz and Fambrough, 1986; Mane et al., 1989). However, we have no evidence that gp65 is expressed on the surface of *T. vivax*.

In addition to the network of endocytic organelles, another tubulo-vesicular system has been described in the posterior portion of trypanosomes. These organelles are thought to be involved with the processing of VSG: namely, turnover, export, and degradation (Frevert and Reinwald, 1988; Webster and Grab, 1988; Seyfang et al., 1990). Previous studies using anti-VSG antibodies on ultrathin sections of trypanosomes have demonstrated extensive labeling of tubules and vesicles in the region of the flagellar pocket (Frevert and Reinwald, 1988; Webster and Grab, 1988). In colocalization studies using Tf-gold and anti-VSG antibodies, Webster and Grab (1988) have shown that most of the antibody-labeled organelles do not contain the endocytosed marker, suggesting that the organelles labeled with anti-VSG antibodies are part of a separate pathway that is involved in VSG processing. Observations from the colocalization experiment (Figs. 9 and 10), where not all of the mAb-labeled organelles contained BSA-gold even when colocalization of mAb 4E1 and BSA-gold was maximal (5 min), prompted us to investigate the possibility that gp65 resides in the VSG-containing tubulovesicular organelles, as well as the endocytic organelles described. This would suggest a role for gp65 in two cellular pathways. Therefore, double antibody labeling using mAb 4E1 and RxVSG on ultrathin sections of trypanosomes containing endocytosed BSA-gold was performed and showed that mAb 4E1 and RxVSG did not colocalize to similar organelles within the cell. In agreement with previous studies (Webster and Grab, 1988), we find little colocalization of RxVSG-labeled structures and endocytosed BSA-gold after 2–3 min of endocytosis, suggesting a mechanism for sorting of internalized VSG and endocytosed BSA-gold. Although the VSG coming into the cell cannot be distinguished from the VSG being exported from the cell, we have concluded from this experiment that the gp65-containing organelles have no obvious involvement in VSG processing.

In summary, we describe a 65-kD integral membrane glycoprotein that is localized to tubulo-vesicular structures of an intermediate part of the endocytic pathway in *T. vivax*. The epitope recognized by mAb 4E1 is specific to *T. vivax*, but a comparable protein may be found in the other species of trypanosome, and thus gp65 may be useful as a marker for the further study of endocytosis in other African trypanosomes. The only other markers for endocytosis in trypanosomes described to date are a low density lipoprotein receptor (Coppens et al., 1988, 1991), a 77-kD protein found in coated vesicles of *T. brucei* (Webster and Shapiro, 1990), and a *T. congolense*–specific lysosomal cysteine protease (Mbawa et al., 1991). The need still remains for other markers that will help to delineate the endocytic pathway of trypanosomes and for the elucidation of the role of component proteins, in order to understand the processes on which these parasites are dependent for survival.

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