The Identification, Purification, and Characterization of Two Invariant Surface Glycoproteins Located beneath the Surface Coat Barrier of Bloodstream Forms of Trypanosoma brucei*

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Two new polypeptides, termed ISG70 and ISG80, have been found in Trypanosoma brucei, using enzyme-catalyzed radioiodination techniques. Both are externally disposed integral membrane glycoproteins, containing N-linked carbohydrate chains. No structural homology was detected between ISG70, ISG80, or the variant surface glycoprotein (VSG) when assessed by 1) comparative peptide mapping, 2) immunoprecipitation analysis, and 3) lectin affinity chromatography.

ISG70 occurred in 5.1 x 10^4 copies/cell and has been purified 880-fold from detergent extracts of plasma membranes by a procedure that includes gel filtration, lectin affinity chromatography, and preparative SDSPolyacrylamide gel electrophoresis. ISG70 was present only in bloodstream forms and was specifically detected in six different cloned variants from the Molteno Institute trypanosomal antigen type (MITat) serodeme of T. brucei and from the single cloned variant of the International Laboratory for Research on Animal Diseases trypanosomal antigen type (ILTat) serodeme that was examined.

Rabbits with chronic infections of T. brucei displayed circulating antibodies against ISG70. Both the immunogenicity of ISG70 and its invariant nature suggest that it may be useful in the development of an effective serodiagnostic test. Furthermore, its stage-specific location combined with its invariant nature implies that its function is strictly related to a physiological role required for the parasite's residence in its mammalian host.

The African trypanosomes have enormous medical, veterinary, and economic importance. They also serve as good models of eukaryotic cell function. However, compared with other cells, little is known about their numerous surface macromolecules, which in the case of trypanosomes, lie beneath the VSG or within the partly exposed regions of the flagellar pocket, where they may mediate many of the interesting interactions between host and parasite whose molecular basis remains to be established. In contrast to this situation considerable knowledge is available about the VSG itself (for reviews see Refs. 1–3).

Several early studies identified a series of antigens of unknown function residing on the external surface of the plasma membrane. Several nonvariant surface antigens were restricted to the bloodstream forms of these cells, and limited data indicated that some of these antigens were reached by antibodies bathing live cells (4). Further reports identified an invariant doublet of M, = 22,000 on the cell surface (5) and glycoproteins of M, = 60,000 and 66,000 on the flagellar pocket membrane (6). Further evidence also indicated that the membrane fraction containing these glycoproteins, when used as an immunogen, provided incomplete protection to mice against subsequent challenge with live parasites, whereas an earlier study reported that whole purified plasma membranes provided no such protection in goats or rabbits (7).

A number of specific enzymes has also been localized to the plasma membrane of bloodstream forms of Trypanosoma brucei. For example both adenylate cyclase and the ouabain-sensitive Na^+/K^+-ATPase were found in the plasma membrane (8), whereas about half of the acid phosphatase and acid phosphodiesterase appear to be located on the outer surface of the plasma membrane lining the flagellar pocket (9–12).

Several genes for other possible plasma membrane proteins, termed ESAGs for expression site-associated genes, have been identified in T. brucei and code for amphipathic proteins with typical membrane signal sequences and consensus sites for N-glycosylation (13–15). These proteins are expressed in bloodstream forms (16–21) and metacyclic forms (20) but not in procyclic forms (17). ESAG 4 has been experimentally localized to that portion of the plasma membrane covering the flagellum (22), and sequence data identify it as the Ca^{2+}-activated adenylate cyclase reported previously (23) and now known to function only in bloodstream forms (24, 25). ESAG 8 is a Zn^{2+} finger protein, containing leucine-rich repeats, that is expressed at very low levels (26, 27) and shows homology with the ras-interacting domain of yeast adenylate cyclase. This domain is absent from the Ca^{2+}-regulated adenylate cyclase in T. brucei (19, 27), suggesting that ESAG 8 may be the regulator of the ESAG 4 cyclase. The ESAG family of genes appears to be widely distributed among the African trypanosomes, including brucei, rhodesiense, gambiense, evansi, and equiperdum as well as probably present with some sequence alterations in congoense, vivax, and mega (14, 16, 20, 25) but absent from Trypanosoma cruzi, Leishmania tarantulae, and Crithidia fasciculata (16, 25). A number of genes located outside of the VSG expression sites, termed GRES-

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§ The abbreviations used are: VSG, variant surface glycoprotein; VSG70, membrane-bound form of the variant surface glycoprotein; VSG80, released form of the variant surface glycoprotein; ILTat, International Laboratory for Research on Animal Diseases trypanosomal antigen type; ISG, invariant surface glycoprotein; MITat, Molteno Institute trypanosomal antigen type; PBS, phosphate-buffered saline; BSA, bovine serum albumin; PMSF, phenylmethylsulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis; TBS, 2-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino]ethanesulfonic acid.
AGs for genes related to ESAGs are expressed in both procyclic and bloodstream forms (24, 26, 28). Their protein products represent additional adenylate cyclases with extracellular domains different from that encoded by ESAG 4 and not regulated by Cg+ (22, 25). Recently, a second family of genes, the BSI family, has been identified that may also code for plasma membrane proteins (29).

Three plasma membrane receptors for ligands that circulate in their mammalian host have also been identified in bloodstream forms of T. brucei: the receptor for low density lipoprotein (30), a transferrin-binding protein which is the product of the ESAG 6 gene (31), and possibly a functional EGF receptor (32). Most recently a number of invariant surface glycoproteins or ISGs (33) have been identified on the surface of the bloodstream forms of T. brucei (34, 35). Two ISGs of unknown function have been cloned and sequenced, and their structure has revealed no obvious homology with other membrane glycoproteins (34, 35).

In this report we describe two novel invariant surface glycoproteins that possess extensive reactivity during cell surface radioiodination without similar reactivity during surface radiolabeling with [sithi~nyl-3H]acetimidate. One of these glycoproteins, ISG60, has been partially purified, characterized, shown to be stage-specific, to occur in low copy number, and to be uniformly distributed over the surface of the cell.

**EXPERIMENTAL PROCEDURES**

Materials—Lactoperoxidase (EC 1.11.1.7) from bovine milk, glucose oxidase (EC 1.1.3.4) from Aspergillus niger, trypsin (EC 3.4.21.4) from bovine pancreas, N-chlorosuccinimide, protein A-Sepharose CL 4B concanavalin A-Sepharose 4B, wheat germ agglutinin-Sepharose 6MB, and Ricinus communis agglutinin-agarose were obtained from the Sigma Chemical Company Ltd., Poole, Dorset, United Kingdom. Lactoperoxidase (EC 1.11.1.7) from Flavobacterium meningosepticum was obtained from Boehringer.

Concanavalin A-Sepharose 4B, lentil lectin-Sepharose 4B, wheat germ agglutinin-Sepharose 6MB, and Ricinus communis agglutinin-agarose were obtained from the Sigma Chemical Company Ltd., Poole, Dorset, United Kingdom. N'-Glyc昔anilide (EC 3.2.2.18) from Flavobacterium meningosepticum was obtained from Boehringer. All other chemicals and solvents were obtained from either BDH Chemicals Ltd., Poole, Dorset, United Kingdom or Riedel de Haen.

**Surface Labeling of Trypanosomes**—Cloned variants of T. brucei (MITat 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, and 1.8) were gifts from Dr. G. A. M. Cross and ILTat 1.21 was the gift of Dr. M. J. Turner. Bloodstream forms of these organisms were grown in laboratory rats and purified as described previously (37). Prochylacmic forms of T. brucei were produced by transformation of MITat 1.1 in medium SDM-79 containing heat-inactivated (60 °C, 15 min) fetal calf serum (10%, v/v) and sodium citrate as described by Brun and Schonenberger (38) and then maintained in the same medium.

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**Metabolic Labeling of Trypanosomes**—ISG60 was synthesized as described previously (36).

Purification of ISG60 from bloodstream forms of T. brucei was accomplished using the procedure described below. All steps were carried out at 4 °C unless specified otherwise. A suspension of freshly harvested trypanosomes (2×107 cells) was mixed with surface-radioiodinated cells (4×107) to provide a tracer or marker for ISG60 during its purification. The mixed suspension was centrifuged (10,000 g, 2 min), and the resulting pellets and supernatants analyzed by SDS-PAGE.

**Purification of Plasma Membranes**—Plasma membranes were isolated from whole bloodstream forms of T. brucei. The iodination reaction was terminated by the addition of ice-cold phosphate buffer (0.5 ml) containing cysteine (1 mM), PMSF (0.5 mM), leupeptin (50 μg/ml), and EDTA (1 mM), followed by washing the cells four times by centrifugation (500 g, 30 s) and resuspension in this same medium. IODO-GEN-catalyzed surface radiiodination was performed essentially as described by Markwell and Fox (40).

**Measurement of [3H]Covalency Linked to Protein**—Samples (5–50 μl) of radiiodinated cells or of subcellular fractions derived from radiiodinated cells were extracted into 20 volumes of a mixture of chloroform/methanol (2:1, v/v), and the resulting emissions were filtered under negative pressure through Whatman GF/C glass fiber filter disks. The filters were then washed thoroughly with chloroform/methanol (10 ml/filter), dried, and their content of radioactivity measured in a Packard γ spectrometer. The recovery of radiiodinated protein on the filters was linear within the range 0.025–100 μg of protein expressed as a concentration of 0.2 to 20 μg/ml, with all proteins being detectable when the detergent present or of the duration of extraction or the temperature of the solvent.

**Mild Trypsin Treatment of Radiiodinated Cells**—A minor modification of the method of Taylor and Cross (41) was used. Freshly radiiodinated cells (109/ml) in phosphate buffer were incubated with trypsin (40 μg/ml) at 37 °C for 15 min prior to the addition of an equal volume of ice-cold phosphate buffer, containing soybean trypsin inhibitor (80 μg/ml). The cells were centrifuged (9,000 × g for 2 min) and the resulting pellets and supernatants analyzed by SDS-PAGE.

**Osmotic Lysis of Cells**—Cells (109/ml) in phosphate buffer were incubated with an excess of phosphate buffer, containing leupeptin (50 μg/ml), PMSF (0.5 mM), and EDTA (1 mM) and incubated at 37 °C for 10 min. The cell lysates that resulted were centrifuged (10,000 × g for 2 min), and the pellets (containing crude plasma membranes) and supernatants (containing impure VSG) were analyzed by SDS-PAGE.

**Preparation of Detergent Lysates and Immunoprecipitates**—The general approach of Anderson and Blobel (42) was used. Detergent lysates were made by mixing freshly surface radiiodinated cells (109/ml) with an equal volume of a solution of SDS (4%, w/v) in phosphate buffer at 25 °C. The cell pellets were boiled for 5 min in the same buffer, containing a 4-fold excess of phosphate buffer, containing Triton X-100 (2.5%, v/v), PMSF (0.5 mM), leupeptin (50 μg/ml), and EDTA (1 mM) and then maintained at 0 °C for an hour, followed by centrifuging at 9,000
x g for 5 min. Immunoprecipitations were performed by mixing samples (0.1 ml) of the supernatants of centrifuged detergent-lysed cells (10^7 with 10-20 μl (determined by titration in each case) of the appropriate antiserum or IgG fraction of the antiserum. Antibody-treated lysates were then mixed with a slurry (1:1, w/v) of protein A-Sepharose CL 4B in phosphate buffer in a ratio of 5 volumes of filtrate to 1 volume of serum or serum fraction used. The mixture was agitated for 2 h before centrifuging (9,000 x g) for 30 s to sediment the resin-bound immune complexes. The sedimented resin was washed in sequence by centrifuging and resuspending two times in Tris-Triton buffer (Tris-HCl, 50 mM, adjusted to pH 7.5; 100 mM NaCl, 1% Triton X-100, 0.5% TPI, 0.1% TPI; 0.1% TPI; and 0.1% TPI; 0.1% TPI, 0.1% TPI, 0.1% TPI, 0.1% TPI, 0.1% TPI), buffer supplemented with 0.5 M NaCl and, finally, twice in Tris-Triton buffer omitting the Triton X-100. The immune complexes were eluted from the washed resin by boiling in SDS-PAGE sample buffer for 2 min.

Competitive Immunoprecipitation Assay for ISG7—Samples (5 μl) of anti-ISG7 serum were incubated with portions (175 μl) of a detergent lysate of surface-radioiodinated bloodstream forms (10^7/ml) of T. brucei (MTat 1.1). Sufficient detergent lysate was added to half-saturate the antibody with 125I-labeled ISG7. This lysate-antibody mixture was further mixed with a 15-fold excess (unlabeled cells/labeled cells) of either unlabeled bloodstream forms (MTat 1.1) or unlabeled procyclic forms, originally derived from bloodstream forms of MITat 1.1, and then incubated overnight at 5 °C, prior to recovery of the immune complexes (see previous section).

Radioactivity in the complexes was measured using a Packard gamma counter. In Lectin Affinity Chromatography of ZSGs—Preparations of radioiodinated ISG7 and ISG9 in deoxycholate extracts of crude plasma membranes were chromatographed on small columns (0.5-1 ml bed volume) of a variety of different lectins, including concanavalin A, lentil lectin, wheat germ agglutinin, and Ricinus communis agglutinin, under the same conditions described for the purification of 125I-labeled ISG7 on concanavalin A-Sepharose. The competing sugars (0.2 M in each case) used for the elution of bound glycoproteins were α-methylmannoside (concanavalin A-Sepharose and lentil lectin-Sepharose), N-acetylglucosamine (wheat germ agglutinin-Sepharose), and the lectin-specific carbohydrates (R. communis agglutinin-Sepharose). The eluates were dried and subjected to SDS-PAGE and autoradiography.

Deglycosylation of ISG7—Two alternative procedures were used. In the first procedure 125I-labeled ISG7 present in Triton X-100 extracts of crude plasma membranes were partially purified on concanavalin A-Sepharose prior to treatment with N-glycoproteinase (4). Crude plasma membranes, derived from osmotically lysed surface radioiodinated trypanosomes (10^7 cells), were extracted into 1.0 ml of Triton X-100 (1%, w/v) in Tris-HCl buffer (20 mM, pH 7.5) supplemented with NaCl (100 mM). Following centrifugation (9,000 x g, 5 min), the clarified extract was incubated (1 h, 5 °C) with 0.1 ml of concanavalin A-Sepharose, and the last two washings were pooled and the resin bound immune complexes were eluted with SDS-PAGE sample buffer exactly as described by Lischwe and Ochs (48). Finally, the gel slices were placed in the sample wells of a 15% (w/v) polyacrylamide SDS-PAGE gel and electrophoresed. The completed peptide maps were visualized by autoradiography.

Western Blotting—Transfer of proteins from SDS-PAGE gels to nitrocellulose membranes and subsequent incubation with antibody were performed as described previously (49) with the exception that the primary antibodies were detected using a 1:1,000 dilution of either 125I-coupled goat antimouse IgG (40) or 125I-coupled rabbit antiserum (41) rather than using 125I-coupled protein A. The antigen-antibody complexes were detected on washed membranes following their incubation in a solution of 4-chloro-1-naphthol (0.05%, w/v), hydrogen peroxide (0.015%, w/v) and methanol (0.17%, v/v) in phosphate-buffered saline as described by Towbin et al. (50).

Raising Antiserum—Rabbit anti-VSG (MTat 1.1) and rabbit anti-VSG bloodstream form trypanosomal plasma membranes (MTat 1.1) were prepared using the general procedures for the preparation of polyclonal antisera described by Owen (51). Rabbits were primed with either 150 μg of purified MITat 1.1 VSG, or 3 μg of purified MITat 1.1 VSG/μl in complete Freund's adjuvant (further diluted with equal volumes of saline) at 2-week intervals. A booster injection was given 2 weeks following the initial injection. Rabbit anti-ISG70 antiserum was prepared by initially inoculating microgram quantities of purified ISG70 in phosphate buffer directly into the popliteal lymph nodes as described by Siegel et al. (52). Otherwise conventional procedures were followed for the production of rabbit anti-ISG10 antiserum.

Hyperimmune antiserum was collected from a rabbit that had a chronic parasitemia approximately 3 months after an initial inoculation of the rabbit with 10^5 bloodstream forms of T. brucei (MTat 1.2).

Mouse anti-ISG70 antiserum was prepared by repeated (×3) intraperitoneal immunization of male outbred mice with homogenized SDS-PAGE gel slices containing microgram quantities of purified ISG70 in phosphate buffer. Antibody was subsequently purified from the rabbit anti-ISG70 antiserum on protein A-Sepharose.

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Immunofluorescence Microscopy—Bloodstream forms of T. brucei (MTat 1.1) were fixed in 2% paraformaldehyde in PBS at room temperature for 20 min and immediately neutralized by the addition of Tris-HCl (25 mM, pH 8.0) and methanol (95% v/v) in PBS at room temperature for 10 min. The fixed bloodstream forms were then incubated with the antibody of interest for 30 min. Following incubation, the slides were washed three times in PBS and air-dried. Nonspecific protein binding sites were blocked by incubation of the slides with PBS, the cells were treated for 14 h at 4 °C with the appropriate antibody, and then incubated overnight at 4 °C with anti-VSG (MTat 1.1) serum. Following rinsing in PBS, the slides were washed with PBS/BSA, and rehydrated in a solution of 4-chloro-1-naphthol (0.05%, w/v), hydrogen peroxide (0.015%, w/v) and methanol (0.17%, v/v) in phosphate-buffered saline as described by Towbin et al. (50).

Coomassie Blue-stained SDS-PAGE gels and Western blots of plasma membranes, derived from osmotically lysed surface radioiodinated bloodstream forms (10^7/ml) of T. brucei (MTat 1.1). Sufficient detergent lysate was added to half-saturate the antibody with 125I-labeled ISG7. This lysate-antibody mixture was further mixed with a 15-fold excess (unlabeled cells/labeled cells) of either unlabeled bloodstream forms (MTat 1.1) or unlabeled procyclic forms, originally derived from bloodstream forms of MITat 1.1, and then incubated overnight at 5 °C, prior to recovery of the immune complexes (see previous section).

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appropriate antiserum (anti-ISG70, preimmune) diluted 1:50 in PBS/BSA. The slides were washed with Tris buffer (50 mM Tris, pH 8.0, 150 mM NaCl) prior to treatment with alkaline phosphatase-conjugated anti-rabbit immunoglobulin for 1.5 h at room temperature diluted 1:500 in PBS/BSA. Invariant Surface Glycoproteins of T. brucei were then prepared for staining with the alkaline phosphatase substrate Vector Red according to the manufacturer’s instructions. Prior to mounting in glycerol, the slides were washed with ethanol. Cells were viewed with a Nikon optiphot fluorescence microscope equipped with a x 100 oil immersion Planapo objective.

**RESULTS**

**Cell Surface Radiolabeling Detects Two Previously Unidentified Antigens on the External Surface of Bloodstream Forms of T. brucei—Cloned bloodstream forms of T. brucei (MITat 1.1) were surface-radiolabeled with either [isethionyl-3H]acetimidate or 125I and then subjected to one- and two-dimensional polycrylamide gel electrophoresis followed by autoradiography. The VSG and two other invariant surface glycoproteins (ISG70 and ISG44) of M, = 70,000 and 64,000, respectively, that were not detectable by Coomassie Blue or silver staining were radiolabeled by enzymatic radioiodination while only the VSG was radiolabeled by radioacetylation (Fig. 1A). It is important to note that the M, = 67,000 marker was bovine serum albumin, which is known to migrate in SDS-PAGE at an anomalously high molecular weight value and did not lie on the calibration curve constructed from the remaining markers in this study. Consequently, the M, values of the ISGs and the VSG were calculated without reference to this particular marker. Longer exposure of the autoradiograms revealed the presence of a small number of additional radioiodinated proteins in the range of M, = 45,000–150,000. However, these minor components have not been characterized further in this present study. It was interesting to note that the intensity of labeling by radioiodination of ISG70 in particular was almost as intense as that observed for the VSG despite the large excess of VSG as assessed by Coomassie Blue staining. It seems likely that this observation reflects the unusual aspect of the structure of ISG70 such as a relatively high tyrosine content of that portion of the poly-peptide exposed to the outer surface of the cell.

**Neither ISG70 nor ISG44 is Structurally Related to the VSG—** Following SDS-PAGE of whole cells (MITat 1.1), the bands of radiiodinated ISG70, ISG44, and VSG were cut from the gel, and each protein was peptide mapped using N-chlorosuccinimide (15 mM) for 30 min at 20 °C prior to repeat SDS-PAGE followed by autoradiography. The identity of the samples is ISG70 (lane a), ISG44 (lane b), VSG (lane c). The molecular weight marker proteins were β-galactosidase (M, = 116,000), phosphorylase b (M, = 97,400), bovine serum albumin (M, = 67,000), ovalbumin (M, = 45,000), and carbonic anhydrase (M, = 29,500).

Fig. 1. **Surface radiolabeling and peptide mapping of ISG70, ISG44, and the VSG.** Surface-radiolabeled cells (approximately 2 x 10^7) were subjected either to SDS-PAGE (A) followed by staining with Coomassie Blue, drying, and autoradiography. A, track 1 shows the results of radiolabeling with [isethionyl-3H]acetimidate, whereas track 2 shows the results of enzymatic radioiodination. The faint band (M, = 120,000) in track 1 is a cross-linked dimer of the VSG produced by a side reaction during formation of the labeled imidoesters (68–70). B, slices (1 mm) of SDS-polyacrylamide gels that contained ^35S-labeled ISG70, ^125I-labeled ISG44, or ^125I-labeled VSG, (MITat 1.1) were prepared as described under “Experimental Procedures” and then treated with N-chlorosuccinimide (15 mM) for 30 min at 20 °C prior to repeat SDS-PAGE followed by autoradiography. The identity of the samples is ISG70 (lane a), ISG44 (lane b), VSG (lane c). The molecular weight marker proteins were β-galactosidase (M, = 116,000), phosphorylase b (M, = 97,400), bovine serum albumin (M, = 67,000), ovalbumin (M, = 45,000), and carbonic anhydrase (M, = 29,500).

In a second independent approach antiseraum raised against purified VSG (MITat 1.1) precipitated only the VSG and did not precipitate either ISG70 or ISG44 from detergent extracts of whole cells. In addition immunoprecipitation of ISG70, radiolabeled with [35S]methionine in vivo, from a detergent lysate prepared from bloodstream forms of T. brucei demonstrated unequivocally that ISG70 was a genuine trypanosomal protein (Fig. 2) rather than a host protein adsorbed to the surface coat of the cell. Moreover, antisera from a rabbit with chronic active trypanosomiasis that had sustained repeated cycles of antigenic variation initiated with live cells of a different variant (MITat 1.2) also precipitated both ISGs but failed to recognize the VSG. These results demonstrate the structural differences between the ISGs and the VSG from both homologous and heterologous variants of T. brucei and indicate the capacity of ISGs to elicit an immune response independent of the VSG during the course of a natural infection.

**Purification of ISG44—** Essentially all of the ISG44 present in whole cells was recovered in the initial crude membrane fraction and after extraction of the membranes with deoxycholate (Table I). The majority of the VSG was released into the supernatant following rupture of the cells as reported previously (10). However, significant amounts of VSG contaminated the membrane fraction (see Fig. 3A) despite extensive washing with high salt buffers and probably represents material trapped within membrane vesicles that were created during the osmotic lysis procedure. Removal of this contaminated VSG was usually achieved during the final preparative SDS-PAGE step (Fig. 3B), but alternative methods such as chromatography on anti-VSG-Sepharose were also found to be effective.

Gel-filtration of the deoxycholate extract of membranes
(not shown) separated ISG\textsubscript{70} from the bulk of the membrane proteins which chromatographed as a large skewed peak close to the excluded volume of the Ultrogel AcA 34 column (exclusion limit $> M_\text{r} = 10^6$). Interestingly, ISG\textsubscript{70}, which migrates with an apparent $M_\text{r} = 70,000$ on SDS-PAGE gels (Fig. 3B), eluted in the same volume as IgG ($M_\text{r} = 150,000$) during gel filtration (not shown) and was clearly separated from the VSG which migrates as a monomer ($M_\text{r} = 60,000$) in the presence of deoxycholate (55). One possible explanation of this result is that native ISG\textsubscript{70} forms dimers in solution. Other possibilities such as the formation of disulfide-linked aggregates containing ISG\textsubscript{70} are less likely, since we have detected no difference in the mobility of this glycoprotein under either fully reduced or nonreduced conditions when assessed by SDS-PAGE (not shown).

SDS-PAGE analysis of the ConA-purified material (Fig. 3, track e) shows that a faint Coomassie Blue-stained band in the same position as \textsuperscript{125}I-labeled ISG\textsubscript{70} was clearly visible. ISG\textsubscript{64} was also detected in this same fraction by autoradiography (Fig. 3, track f) and appears to copurify with ISG\textsubscript{70} throughout the purification procedure up to the final step of preparative SDS-PAGE. However, ISG\textsubscript{64} was not easily visible by Coomassie Blue staining due to the presence of relatively large quantities of additional proteins, including the VSG, which migrates in the same position as ISG\textsubscript{64} during SDS-PAGE (Fig. 3, track e).

Following preparative SDS-PAGE, a sample of the material recovered from the gel corresponding to ISG\textsubscript{70} was re-electrophoresed on an analytical SDS-PAGE gel (Fig. 3B). The purified ISG\textsubscript{70} migrated as a diffuse band as visualized by Coomassie Blue staining. Analysis of the radioactivity profile confirmed that most or all of this stainable material was labeled with \textsuperscript{125}I and further revealed that the stained band consisted of two or more very closely migrating species. Similarly, ISG\textsubscript{70} metabolically labeled with \textsuperscript{[35}S\textsuperscript{]}methionine also migrated as a doublet (Fig. 2). The possibility that these preparations of ISG\textsubscript{70} contain low levels of other polypeptide species must also be considered.

The final yield of purified ISG\textsubscript{70} was low (6\%, Table I) but sufficient for the production in mice of small quantities of specific antisera. The major losses of ISG\textsubscript{70} occurred mainly during the two column chromatography steps and may have been due to nonspecific adsorption of ISG\textsubscript{70} onto the relatively large surface area of the matrix. A similar type of loss was reported by Fox \textit{et al.} (56) during the purification of the VSG-specific glycosylphosphatidylinositol phospholipase C from \textit{T. brucei}.

\textbf{ISG\textsubscript{70} Cell Copy Number—}Each cell was calculated to contain $5.1 \times 10^4$ copies of ISG\textsubscript{70} based upon the data in Table I. Consequently, ISG\textsubscript{70} accounts for 0.12\% of the total cell protein, which is 190-fold less than the VSG.

\textbf{Isoelectric Points of ISG\textsubscript{70} and ISG\textsubscript{64}—}The isoelectric points of ISG\textsubscript{70} and ISG\textsubscript{64} were determined by two-dimensional electrophoresis (isoelectric focussing/SDS-PAGE) followed by Western blotting and were found to be 6.8 and 7.2, respectively.

\textit{Both ISG\textsubscript{70} and ISG\textsubscript{64} Are Located at the Outer Surface of the Plasma Membrane—}Initial experiments demonstrated that radiolabeled ISG\textsubscript{70} and ISG\textsubscript{64} were associated exclusively with the particulate fraction of the cell, whereas the VSG was released into the soluble fraction, following osmotic rupture of the cells (Fig. 4, tracks e-f) due to activation of the VSG releasing enzyme under these conditions (39). Subsequent

\begin{table}[h]
\centering
\caption{Recovery of ISG\textsubscript{70} at each stage of purification}
\label{table1}
\begin{tabular}{lcccc}
\hline
Stage of purification & Protein recovered & \textsuperscript{125}I-Labeled ISG\textsubscript{70} recovered & \textsuperscript{125}I-Labeled ISG\textsubscript{70} specific activity & Purification factor & Yield \\
& mg & cpm $\cdot 10^4$ & cpm $\cdot 10^5 \cdot mg^{-1}$ & & \\
\hline
Whole cells & 63.0 $\pm$ 16.0 (4) & 2.00 $\pm$ 0.60 (3) & 3.40 $\pm$ 0.6 (4) & 1.0 & 100 \\
Membranes & 17.0 $\pm$ 3.0 (3) & 1.60 (1) & 9.40 (1) & 2.8 & 80 \\
Deoxycholate extract & 21.0 $\pm$ 6.0 (4) & 1.60 $\pm$ 0.40 (3) & 8.20 $\pm$ 3.0 (4) & 2.4 & 80 \\
Gel filtrate & 5.0 $\pm$ 1.0 (3) & 0.60 $\pm$ 0.2 (3) & 11.0 $\pm$ 1.0 (3) & 3.2 & 30 \\
ConA eluate & 0.060 $\pm$ 0.010 (3) & 0.120 $\pm$ 0.04$^*$(3) & 21.0 $\pm$ 80.0 (3) & 62.0 & 6 \\
Preparative SDS-PAGE & 0.004 $\pm$ 0.001$^*$ (2) & 0.120 $\pm$ 0.04 (3) & 3000.00 $\pm$ 1200.0 (2) & 880.0 & 6 \\
\hline
\end{tabular}
\begin{flushleft}
\textsuperscript{*}This value was determined from the ISG\textsubscript{64} band cut from the subsequent preparative SDS-PAGE gel that had been loaded with the combined ISG\textsubscript{70} fractions from the ConA step rather than from a separate analytical SDS-PAGE gel as used for assaying each of the previous steps. The assumption was made that the recovery of ISG\textsubscript{70} would be the same on analytical and preparative SDS-PAGE gels.
\end{flushleft}
\begin{flushleft}
\textsuperscript{1}In this case the protein concentration was estimated by densitometric scanning of Coomassie Blue-stained SDS-PAGE gels.
\end{flushleft}
\end{table}
many times that preparations of cells which showed high intensity labeling of ISG4 in pellets of water-lysed cells also showed high intensity labeling of this protein in purified membranes after various treatments and multiple washes. Conversely, when the initial labeling of ISG4 was low, the same low content of labeled ISG4 was likewise found in the purified membranes. Furthermore, subjecting surface-radio-labeled cells to a second step of cell purification on a DEAE-cellulose column (see “Experimental Procedures”) failed to alter the relative proportions of ISG4, ISG70, and the VSG. Finally, repeated washing of membranes with 0.5 M KCl did not remove detectable amounts of ISG4 or ISG70, whereas detergents readily solubilized both proteins, making it unlikely that either of these proteins were loosely attached peripheral trypanosomal proteins or adsorbed host proteins.

Mild trypsin treatment of whole intact radioiodinated cells removed essentially all of the radioactivity associated with ISG70, ISG4, and the VSG (Fig. 4, track c). Even following trypsin treatment under the conditions used in these experiments, the cells remained fully motile and phase dense as assessed microscopically, leading to the inevitable conclusion that the cells also remained intact. Consequently, this result also supported a second conclusion, based on the results of the surface labeling and cell fractionation experiments, that both ISGs in common with the VSG were located at the external surface of the cell. SDS-PAGE analysis of the material released by trypsin (Fig. 4, track d) revealed the presence of only a major doublet of radioactive Coomassie-stained bands of \( M_r = 45,000-50,000 \) which were identified subsequently as fragments of the VSG by immunoprecipitation with VSG specific antiserum (data not shown). The failure to detect radioiodinated tryptic fragments of either ISG70 and ISG4 suggests that these fragments may have been too small to be retained within the polycrylamide gel during fixation and staining.

Both ISG70 and ISG4 are N-Glycosylated Glycoproteins—
The lectin binding affinities of radioiodinated ISGs present in deoxycholate extracts of purified plasma membranes (VSG absent in these extracts) were studied using concanavalin A-Sepharose (ConA), wheat germ agglutinin-Sepharose (WGA), R. communis agglutinin-Sepharose (RCA120), and lentil lectin-Sepharose (LL). Comparison of the elution profiles obtained in each case demonstrated that each of the lectin columns with the exception of lentil lectin bound significant amounts of ISGs in the order of decreasing affinity: ConA > RCA120 ≈ WGA >> LL. A typical elution profile for one of these columns, the ConA affinity column, is shown in Fig. 5. Interestingly, large quantities of ISGs failed to bind any of the lectins as evidenced by the high levels of radioactive ISG eluting with the flow-through fraction in each case, despite the fact that the ISG concentration was well below the binding capacity of the immobilized lectins. In addition, elution of bound ISGs from columns of ConA and elution of crude Ultrogel-filtered ISG70 from ConA resulted in a broad elution profile which appeared to consist of more than one distinct peak.

These results suggested the presence of carbohydrate microheterogeneity in one or both ISGs. Such carbohydrate microheterogeneity would be expected to quantitatively affect lectin binding but not to influence the ability of endoglycosidase F to remove all N-linked glycosyl chains (Fig. 6). Quantitative analysis of the lectin chromatography data revealed differences in the affinity of ISG70 and ISG64 for three of the four lectin columns. For instance ISG70 bound to ConA, RCA120, and WGA (in the order of decreasing affinity ConA > WGA ≈ RCA120), whereas ISG64 bound only to ConA.

Treatment of ISG70 and ISG64 with N-glycopeptidase F destroyed the ability of both glycoproteins to bind ConA and reduced the apparent molecular mass of ISG70 by 8,000 daltons to M, $= 62,000$ as assessed by SDS-PAGE (Fig. 6). Either there was no change in the apparent molecular weight of ISG64 or it comigrated with ISG70 following treatment with N-glycopeptidase F. These latter results indicate that both ISGs contain N-linked sugar chains and suggest that ISG70 is more heavily glycosylated than ISG64.

**ISG70 is a Common Invariant Antigen of Bloodstream Forms of T. brucei**—The possibility that ISG70 was present in a number of different variant populations of T. brucei other than MITat 1.1 was tested by immunoprecipitation from detergent lysates prepared from seven different cloned variants, MITat 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, and ILTat 1.21. The antisera used in these experiments was raised in mice against highly purified (880-fold) ISG70. The antisera proved to be specific for ISG70 and precipitated radiolabeled ISG70 from extracts of five of the six variants of the MITat serodeme tested in this particular experiment and from the single variant of the ILTat serodeme available in our laboratory (Fig. 7). In the one case where little or no ISG70 was precipitated (MITat 1.7), the glycoprotein was detected readily by Western blotting at levels that were similar to those found in both MITat 1.1 and ILTat 1.21 where immunoprecipitation was not a problem. These results suggested that the efficiency of radioiodination of ISG70 in intact cells rather than the level of its expression may differ between different variants. These data also do not eliminate the somewhat unlikely possibility that ISG70 is expressed but not incorpor-
Two novel and previously undescribed invariant surface glycoproteins of \( M, 70,000 \) (ISG\(_70\)) and \( 64,000 \) (ISG\(_{64}\)) that were detected during the surface labeling of bloodstream forms of \( T. \) \( brucei \) with \( ^{125}\)I have been characterized.

The discovery of ISG\(_70\) and ISG\(_{64}\) by cell surface radioiodination was somewhat surprising, since previous studies by other workers who used a variety of different surface labeling reagents, including [\( ^{35}\)S]methionine sulfone methylphosphonate (57), galactose oxidase/Na\(_2\)B\(_3\)H\(_4\) (58), \([\text{S}-^{3}H\]acetylimidate (39), as well as the lactoperoxidase/Na\(_{125}\)I technique (59), detected the presence of only the VSG. However, it appears that the two ISGs are particularly susceptible to iodination. The basis for the apparently selective and extensive reactivity of ISG\(_70\) and ISG\(_{64}\) with \(^{125}\)I under the conditions of the present study was not established with complete certainty but is thought to reflect some unusual aspect of the structure of these glycoproteins, such as a high tyrosine content. This conclusion is supported by the finding that the majority (81\%) of the recovered radioactivity in \(^{125}\)I-labeled ISG\(_{70}\) was found in iodotyrosine residues following enzymatic digestion of the protein and separation of the released amino acids by thin layer chromatography. No other discrete spots of radioactivity on the chromatogram were found. This result eliminates the possibility that any histidine residues were labeled as has been observed frequently when using more vigorous iodination protocols under oxidizing conditions and also eliminates the further possibility that some non-amino acid component of the mature processed protein, such as a carbohydrate or lipid residue that could be iodinated was labeled. Furthermore, the extensive reactivity of the ISGs was not dependent upon the use of the lactoperoxidase/glucose oxidase method for surface radiolabeling, since equally high levels of iodination were observed when radiolabeling

### Table II

| Source of competing unlabeled antigen | \(^{125}\)I-Labeled ISG\(_{70}\) precipitated |
|--------------------------------------|------------------------------------------|
| None                                 | \( 1590 \pm 257 \) cpm                   |
| Bloodstream forms                     | \( 443 \pm 150 \) cpm                    |
| Procyclic forms                       | \( 1780 \pm 383 \) cpm                   |
plasma membrane proteins of trypanosomal origin and not adsorbed host proteins. First, both proteins remain associated with the cells following passage through DEAE-cellulose (pH 8.0, Kreb-Ringer phosphate buffer) during both the initial separation from host elements as well as subsequent to a second identical chromatography step following a buffer wash. Second, both proteins are associated with the particulate fraction following osmotic lysis of the cell and none can be detected in the supernatant fraction. Third, both proteins copurify with plasma membranes from the initial crude pellet following cell lysis and remain with the purified plasma membranes following several high salt (0.5 M KCl) washes. In fact the mildest procedure that removes both proteins from purified membranes is extraction with neutral detergent (1% Nonidet P-40, not shown). Fourth, both proteins are highly immunogenic in rabbits as evidenced by the presence of circulating antibodies in animals infected with live trypanosomes and the absence of such antibodies in noninfected animals. Fifth, both proteins can be immunoprecipitated from detergent extracts of surface $^{125}$I-labeled parasites using either hyperimmune sera from parasitic rabbits or sera raised against highly purified trypanosomal plasma membranes but not when using control sera from uninfected and immunized animals. Sixth and last, ISG$_{70}$, which has been most studied and characterized, can be immunoprecipitated from detergent extracts of $[^{35}]$S$_{methionine}$ biosynthetically labeled parasites. This last experiment has not yet been carried out in the case of ISG$_{4}$, which at present is less well characterized than ISG$_{70}$. Certainly all of the existing evidence indicates that both of these ISGs are integral plasma membrane proteins of trypanosomal origin.

Characterization of the ISGs demonstrated a number of important similarities and differences between ISG$_{70}$ and ISG$_{4}$. First, both of the ISGs were relatively minor cellular components; for example each individual cell was estimated to contain between $10^6$ and $10^7$ copies of ISG$_{70}$, whereas there are over $10^7$ copies of the VSG (49). Second, both ISGs displayed properties that are typical of integral membrane proteins. Third, peptide mapping and differential immunoprecipitation experiments revealed that both ISGs had unique peptide maps and contained unique immunological determinants; neither ISG showed any similarity of primary structure with each other or with the VSG.

Both ISG$_{70}$ and ISG$_{4}$ were found to contain N-linked carbohydrate chains that were sensitive to cleavage by N-glycopeptidase F. Furthermore, these N-linked carbohydrate chains were responsible for the strong affinity for ConA that was characteristic of both glycoproteins. ISG$_{70}$ appears to be much more heavily glycosylated than ISG$_{4}$, based on the finding that its apparent molecular weight shifted by approximately 8,000 following deglycosylation, whereas little or no shift was apparent for ISG$_{4}$. Consequently, it may be estimated that ISG$_{70}$ contains approximately 11% N-linked carbohydrate by weight, which is comparable with that found for the VSG (60). However, it is interesting to note that the N-linked carbohydrate chains of ISG$_{70}$ may not be freely accessible from the exterior of intact cells where they are probably covered by the VSG. Certainly, the carbohydrate chains of the ISGs are not cleaved when whole cells are treated with N-glycopeptidase F (not shown), but they are cleaved when the purified glycoproteins are treated. In addition the carbohydrate composition of the two ISGs was different. This conclusion was shown by the finding that only ISG$_{70}$ bound in any amount to either wheat germ agglutinin or RCA$_{120}$. The fact that not all of the ISG$_{70}$ bound to these two lectins further reveals that ISG$_{70}$ is differentially glycosylated and

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1. D. G. Jackson, H. J. Windle, and H. P. Voorheis, unpublished observation.
demonstrates considerable carbohydrate microheterogeneity.

The marked affinity of ISG₀ for ConA provided a convenient means for the purification of this glycoprotein on a small scale. However, it was necessary to minimize the number of steps used in the purification procedure because of the losses of ISGs encountered, particularly during column chromatography. Extensive losses of other membrane proteins from T. brucei during their purification have been reported (56) and probably results from the marked hydrophobic character of these proteins. The purified ISG₀ migrated as a broad diffuse band on analytical SDS-PAGE gels due probably to the carbohydrate microheterogeneity detected by the lectin binding experiments. However, the possible presence of other unrelated glycoproteins cannot be eliminated completely at this stage.

ISG₀ appears to be an invariant surface glycoprotein, because it was detected unchanged in eight different variants from the MITat serodeme as well as in the only variant from the ILTat serodeme of T. brucei that was tested. In contrast to the results from all of the bloodstream forms examined, both ISG₀ and ISG₆₄ could not be detected in procyclic forms of the parasite, leading to the conclusion that these ISGs are stage-specific proteins restricted to bloodstream forms. Alkaline phosphatase does not copurify with ISG₀. This conclusion is also supported by the results from all of the bloodstream forms examined, whereas ISG₆₄ contains approximately 12% carbohydrate, whereas ISG₀ contains 12% carbohydrate (34). The issue of the relationship between these proteins will be resolved once sequence information is available. These studies are currently in progress.

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Invariant Surface Glycoproteins of T. brucei

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