A Soluble Secretory Reporter System in Trypanosoma brucei

STUDIES ON ENDOPLASMIC RETICULUM TARGETING*

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A homolog of the endoplasmic reticulum (ER) hsp70 protein, binding protein (BiP), from the parasitic protozoan Trypanosoma brucei (Bangs, J. D., Uyetake, L., Brickman, M. J., Balber, A. E., and Boothroyd, J. C. (1993) J. Cell Sci. 105, 1101-1113) is further characterized. In co-precipitation experiments, BiP transiently associates with newly synthesized secretory proteins, including variant surface glycoprotein (VSG), confirming its role as a molecular chaperone. To study the molecular signals targeting BiP to the ER, we have developed soluble secretory reporters for expression in transformed procyclic trypanosomes. Deletion of the BiP C-terminal tetrapeptide (MDDL) and the glycosylphosphatidylinositol-anchor addition sequence of VSG converts these proteins to secreted forms. Attachment of MDDL to VSG results in intracellular retention confirming that MDDL is a trypanosomal ER localization signal. Secretion of both reporters is inefficient, but further truncation of the BiP C-terminal peptide-binding domain allows quantitative export (t½ – 1 h) of the N-terminal ATPase domain (BiPN), consistent with the conserved domain structure of hsp70 proteins. This is the first demonstration of soluble protein secretion in African trypanosomes. Using the BiPN reporter, the sequence specificity of C-terminal tetrapeptide retention signals in trypanosomes is analyzed and found to be similar to higher eukaryotes. These results indicate that the basic signals mediating protein targeting to the ER lumen are conserved throughout the wide range of eukaryotic evolution.

African trypanosomes, parasitic protozoa of the order Kinetoplastida, represent one of the most ancient of all eukaryotic lineages (1). They have a life cycle that alternates between an insect vector, tsetse flies, and mammalian hosts, in which they cause serious disease. Considerable effort has been made in the study of trypanosome molecular biology resulting in the elucidation of many unusual molecular and biochemical pathways, such as trans-splicing and RNA editing. More recently, interest in the basic cell biology of these organisms has grown, and significant progress has been made in areas such as organelar biogenesis (2) and endocytosis (3). One area, however, that has lagged behind is that of secretion and secretory protein trafficking.

Most of what is known about secretory trafficking in trypanosomes comes from biosynthetic studies of VSG, a homodimeric GPI-anchored protein that is the major surface antigen of the bloodstream stage (4-6). Although other exported plasma membrane proteins are known (7, 8), no soluble secretory polypeptides have yet been identified in Trypanosoma brucei. Export of various "factors" (9, 10), as well as soluble protease activities (11), has all been reported. Unfortunately, none of these have been characterized at the molecular level. All exocytoic and endocytoic trafficking in trypanosomes occurs at an invaginated plasma membrane microdomain called the flagellar pocket (3, 12). Since the opening of the lumen of the pocket to the external environment is restricted, it may be that exit of soluble molecules is not possible. However, fluid phase markers can enter the pocket (13), and secretion of soluble polypeptides does occur in the related trypanosomatids, Trypanosoma cruzi (14) and Leishmania donovani (15, 16). Thus, the absence of soluble secretion in T. brucei most likely reflects the failure to detect such molecules rather than the true biological situation.

The VSG biosynthetic studies suggest that a relatively typical eukaryotic secretory pathway is operative in trypanosomes, and membranous structures morphologically resembling the ER and Golgi can be discerned by electron microscopy (13, 17). However, molecular markers for the secretory subcompartments are few. A trypanosomal homolog of BiP has been characterized, and this serves as an ER marker (18), but no other immunological marker proteins are available for cell biological investigations. Not surprisingly then, little is known about the molecular signals that direct the intracellular trafficking and targeting of the protein components of the trypanosomal secretory pathway.

One of the best documented examples of protein targeting in eukaryotic cells is the localization of soluble proteins, such as BiP, to the lumen of the ER (reviewed in Ref. 19). These proteins have C-terminal tetrapeptide sequences that serve as specific ER localization signals. The consensus tetrapeptide motif is XDEL where the amino acid X varies in a species-specific, and sometimes protein-specific, manner. Typical examples are KDEL in mammals, HDEL in Saccharomyces cerevisiae, DDEL in Kluyveromyces lactis, and ADEL in Schizosaccharomyces pombe (19, 20). All of the aforementioned sequences have been tested functionally in transfection experiments. Sequences in other species such as SDEL in Plasmo-

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The abbreviations used are: VSG, variant surface glycoprotein; GPI, glycosylphosphatidylinositol; ER, endoplasmic reticulum; BiP, binding protein; PBS, phosphate-buffered saline; pBSIISK, Bluescript II SK-; PARP, procyclic acidic repetitive protein; CAT, chloramphenicol acetyltransferase; Neo, neomycin phosphotransferase; PCR, polymerase chain reaction; GUS, X-glucuronidase; hsp70, heat shock protein 70 kDa; bp base pair(s).
dium falciparum (21) and KDEL in Giardia lamblia (22) are presumed by analogy to be retention signals. Two putative signals have been found in trypanosomes, MDDL and KQDL at the C termini of BiP (18) and a protein disulfide isomerase homolog (23), respectively. Given the relative divergence of the trypanosomal sequences and the great phylogenetic distance between trypanosomes and species where ER targeting has been assayed directly, it is essential that these sequences be actualized to mediate ER localization.

The main obstacle to the study of protein trafficking in trypanosomes has been the inability to express recombinant genes in these organisms. In recent years this technological hurdle has been overcome (24–26), and stable transformation vectors have been exploited to study both mitochondrial and glycosomal protein targeting (reviewed in Ref. 2). In this report, we use similar techniques to investigate secretory protein trafficking in T. brucei. First, we have confirmed that trypanosomal BiP functions as a molecular chaperone by demonstrating its transient physical interaction with an endogenous secretory protein, VSG. Second, we have developed soluble secretory reporters that are efficiently exported to the extracellular environment when expressed in procyclic cells, explicitly demonstrating for the first time that soluble secretion in African trypanosomes is possible. Finally, we have exploited these recombinant reporters to study the sequence specificity of ER targeting signals. Our results demonstrate that C-terminal tetrapeptides mediate ER localization in trypanosomes and define the sequence specificity of this phenomenon. This work suggests that ER localization occurs by a mechanism that is common to, if not all, eukaryotic organisms.

EXPERIMENTAL PROCEDURES

Growth and Metabolic Labeling of Trypanosomes—Strain 427 (M1-Tar 1 serodeme) bloodstream (clone M1T1.2 expressing VSG221) and procyclic stage (Pro1 cell line derived from clone M1T1.4) trypanosomes were used for all experiments (18). Procyclic cells were grown at 27 °C in TM-P medium (27). For metabolic radiolabeling, procyclic cells were washed twice in PBS and resuspended at 107/ml in growth medium. Bloodstream cells from infected mice (6) were incubated in TM-P medium (27). For metabolic radiolabeling, bloodstream trypanosomes were used for all experiments (18). Procyclic cells were grown at 27 °C in TM-P medium (27). For metabolic radiolabeling, bloodstream trypanosomes were washed four times with wash buffer and once with TEN buffer is 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA) and then dialyzed fetal bovine serum, and then radiolabeled for 5 min (108 cpm). Following radiolabeling, bloodstream trypanosomes were washed twice in PBS and resuspended in TEN buffer. For cell lysate or culture supernatant assays, samples were mixed (1 h, 4°C) and then washed four times with wash buffer and once with TEN. For sequential immunoprecipitation, a primary anti-BiP immunoprecipitate was solubilized in TEN, 1% SDS (50 μl, 65 °C, 10 min) and dialuted with TEN, 1% Nonidet P-40, 0.55% deoxycholate (450 μl). Following centrifugation the supernatant was reprecipitated with anti-VSG (16 h, 4°C) and washed as above. Final precipitates were resuspended in 100 μl Tris-HCl, pH 6.8, 2% SDS, 200 μl dithiotheritol, 20% glycerol, 0.1% bromphenol blue (25 μl), fractionated by 12% SDS-polyacrylamide gel electrophoresis, and analyzed by fluorography. Molecular mass markers (Midrange, Promega, Madison, WI) were used.

The following synthetic deoxyoligonucleotides were used for PCR. Sequences complementary to the target template are in upper case; added sequences are lower case.

**Antibody Preabsorption**—Antibody was preadsorbed to Protein A-Sepharose beads (18), washed with TEN, 1.1% Nonidet P-40, 0.55% deoxycholate (450 μl). Following centrifugation the supernatant was reprecipitated with anti-VSG (16 h, 4°C) and washed as above. Final precipitates were resuspended in 100 μl Tris-HCl, pH 6.8, 2% SDS, 200 μl dithiotheritol, 20% glycerol, 0.1% bromphenol blue (25 μl), fractionated by 12% SDS-polyacrylamide gel electrophoresis, and analyzed by fluorography. Molecular mass markers (Midrange, Promega, Madison, WI) were used.
Fig. 1. pXS2 vector. Panel A, diagram of the pXS2 procyclid stable expression vector. Regions from 5′-3′ are the PARP B2 locus promoter including splice acceptor site (diagonal striped box); the PARP B2 3′ intragenic region (black box); the neomycin phosphotransferase gene (vertical striped box); the tubulin locus 5′-3′ intragenic region (open box). The PARP promoter and intragenic regions are separated by a multicloning site for insertion of reporter genes. The region shown is bounded by the KpnI and SacI sites of pBluescript II KS. Asterisks denote splice acceptor sites. Unique restriction sites are: KpnI (K), XhoI (Xh), ClaI (C), HindIII (H), EcoRV (V), EcoRI (R), Ascl (A), Pad (P), BssXI (B), XbaI (X), SacI (S). Panel B, diagram (not to scale) of wild type (WT) and recombinant reporter genes used in this work. Domain regions are N-terminal signal sequence (diagonal striped box); BIP ATPase domain, (vertical striped box); BIP peptide binding domain (open box); HA9 epitope tag (black box); VSG coding region (white box); VSG GPI anchor sequence (horizontal striped box). The native C-terminal tetrapeptide (MDDL) of wild type (WT) BIP is indicated. Variant C-terminal tetrapeptides used with the BIP reporter series are designated (Xxxx). The full-length trypanosome BiP gene was amplified in two forms, minus the C-terminal MDDL tetrapeptide sequence (lane B71J B73) and minus both the C-terminal tetrapeptide and N-terminal signal sequence (lane B72 J B73). These were cloned into pHA9R using flanking Xmal/HindIII sites to generate the epitope-tagged reporter genes, BIPHA9 and BIPHA9Ass. The 415-amino acid N-terminal ATPase domain of BiP was amplified (lane B93 J B94) and substituted for BiP in BIPHA9 using flanking HindIII/HindIII sites (BIPN). C-terminal modifications of BiP were made by PCR. BIPNMDLD encoding the native C-terminal BIP hexapeptide (QPMDDL) was used generating BIP as template (lane B93 J B64). BIPNAVRG, encoding the C-terminal hexapeptide (QPAVRG), was generated using BIPNMDLD as template (lane B93 J B99). The following derivatives were made with BIPNMDLD or BIPNAVRG as template: BIPNMDL (B93 J B109), BIPNQDL (B93 J B118), BIPNMDD (B93 J B119), and BIPNAVDL (J B93 J B125). The VSG 117 gene was amplified minus the C-terminal GFI addition signal (117gpi) with primers J B79 J B79. 117MDLD was made by replacing the BIPN portion of pBIPNMDLD with the 117gpi cassette.

RESULTS

Functional Analysis of Endogenous BiP—Previous analyses, based on sequence homology and subcellular localization, indicate that we have identified the trypanosomal homolog of the ER hsP70 protein, BiP (18). As such, it would be predicted to act as a molecular chaperone by facilitating the folding and assembly of secretory proteins (33). Therefore, prior to investigation of the molecular signals responsible for intracellular targeting of the trypanosomal BiP homolog, we wished to confirm that it has chaperone function as judged by transient physical interaction with newly synthesized secretory proteins. VSG, the major secretory protein of bloodstream trypanosomes, comprising 10–20% of total cell protein (34), is the ideal choice for such an assay.

Bloodstream trypanosomes were pulse/chase-radioiodinated, and polypeptides were immunoprecipitated from cell extracts with anti-BiP or anti-VSG (Fig. 2A). The total amount of each labeled protein (lanes 1–5, VSG; lanes 6–10, BIP) remained constant throughout the chase period. More importantly, at the earliest chase time, an abundant newly synthesized protein of 415 amino acids (117gpi) with the VSG GPI anchor sequence (horizontal striped box). The native C-terminal tetrapeptide (MDDL) of wild type (WT) BIP is indicated. Variant C-terminal tetrapeptides used with the BIP reporter series are designated (Xxxx). The full-length trypanosome BiP gene was amplified in two forms, minus the C-terminal MDDL tetrapeptide sequence (lane B71J B73) and minus both the C-terminal tetrapeptide and N-terminal signal sequence (lane B72 J B73). These were cloned into pHA9R using flanking Xmal/HindIII sites to generate the epitope-tagged reporter genes, BIPHA9 and BIPHA9Ass. The 415-amino acid N-terminal ATPase domain of BiP was amplified (lane B93 J B94) and substituted for BiP in BIPHA9 using flanking HindIII/HindIII sites (BIPN). C-terminal modifications of BiP were made by PCR. BIPNMDLD encoding the native C-terminal BIP hexapeptide (QPMDDL) was used generating BIP as template (lane B93 J B64). BIPNAVRG, encoding the C-terminal hexapeptide (QPAVRG), was generated using BIPNMDLD as template (lane B93 J B99). The following derivatives were made with BIPNMDLD or BIPNAVRG as template: BIPNMDL (B93 J B109), BIPNQDL (B93 J B118), BIPNMDD (B93 J B119), and BIPNAVDL (J B93 J B125). The VSG 117 gene was amplified minus the C-terminal GFI addition signal (117gpi) with primers J B79 J B79. 117MDLD was made by replacing the BIPN portion of pBIPNMDLD with the 117gpi cassette.

Transformation of Trypanosomes—Mid-log phase (5–10×10^6 cells/ml) procyclid trypanosomes were washed once in PBS and resuspended at 4×10^6/ml in Optimum medium (Life Technologies, Inc.). Qiagen-purified (Qiagen Inc, Chatsworth, CA) pXS2:reporter plasmids were linearized using a unique BstXI site in the 5′-flanking intragenic region cassette, precipitated, washed in 70% ethanol, air-dried, and dissolved at 1.0 mg/ml in Optimum. 0.5 ml of cells and 0.1 ml of linear DNA were mixed in 0.4-mm cuvettes and electroporated (24 V, 1.5 kV) in a BTX600 cell manipulator (BTX Inc., San Diego, CA). Cells were transferred to 10-ml TM-P cultures, and, at 48 h, G418 was added to 50 μg/ml. Stable cell lines typically grew out in 2–3 weeks and were maintained in 25 μg/ml G418.

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fig. 2 co-precipitation of Bip and VSG. Panel A, bloodstream trypanosomes, expressing VSG 221, were metabolically radiolabeled for 5 min and then chased for 15 min. At the indicated times, cell extracts were prepared, and radiolabeled polypeptides were immunoprecipitated with anti-VSG (lanes 1–5, $5 \times 10^6$ cell equivalents) or anti-Bip (lanes 6–10, $10^6$ cell equivalents). Panel B, after a 5-min pulse, radiolabeled polypeptides were immunoprecipitated with anti-VSG (lane 1) or anti-Bip (lane 2). A separate anti-Bip immunoprecipitate was solubilized as described under “Experimental Procedures” and reprecipitated with anti-VSG (lanes 3). All lanes contain $5 \times 10^6$ cell equivalents. Panels A and B, all precipitates were analyzed by SDS-polyacrylamide gel electrophoresis/fluorography. Scans of 3-day (Panel A) and 4-day (Panel B) exposures are presented. The positions of VSG and Bip are indicated. Scales refer to relative molecular mass in kDa. The radiolabeled pool is likely to be associated with VSG. These results indicate that Bip does interact physically with VSG, consistent with its role as a secretory molecular chaperone, and validate its use as a reporter for ER localization studies.

Transformation of Trypanosomes—A vector for the stable transformation of cultured procyclic trypanosomes (pXS2, Fig. 1A) was constructed based on the previous work of others (24–26). Prior to transfection, the vector is linearized at a unique BstXI site in the tubulin intragenic region which targets homologous recombination to the tubulin locus. pXS2 was tested using GUS as an expression reporter gene. A stably transformed GUS cell line was prepared and analyzed in two ways. First, correct targeted integration into the chromosomal tubulin locus was confirmed by Southern analysis. Second, GUS expression was readily detected by both enzymatic assay of cell extracts and by specific immunoprecipitation of GUS polypeptide from metabolically radiolabeled cells (Fig. 3A, lane 1).

Secretion of Epitope-tagged Bip—In order to analyze the molecular signals mediating ER localization in trypanosomes it was first necessary to develop soluble reporters that are efficiently exported. To accomplish this, endogenous polypeptides of the trypanosomal secretory pathway, albeit cell-associated, were adapted as soluble reporters. First, modified Bip genes (Fig. 1B) were generated in which the putative C-terminal ER localization tetrapeptide, MDDL, was replaced with the viral HA9 immuno-epitope to allow discrimination of recombinant from endogenous Bip protein. This reporter was prepared as a secretory form with the native N-terminal signal sequence (BipHA9) and as a cytoplasmic form (BipHA9ss) with the signal sequence deleted. If MDDL is indeed a retention signal, then it is predicted that BipHA9 will be secreted from transformed cells in a soluble manner. Using pXS2, stable cell lines bearing each reporter gene were prepared and analyzed by specific immunoprecipitation of radiolabeled recombinant proteins (Fig. 3). The GUS cell line was used as a control for recombinant Bip expression.

Both Bip reporters are expressed in transformed cells lead-
suggesting that some intracellular degradation may be occurring. No attempt was made to accurately determine the efficiency of export since anti-HA9 antibody does not give quantitative recovery of the recombinant protein (see Fig. 3). Identical experiments were performed in which export of endogenous BiP from untransformed Pro1 cells was monitored for 24 h. These experiments revealed that no endogenous BiP is exported, even in prolonged chases, and that endogenous BiP is extremely stable (τ > 24 h).

Retention of a Heterologous Reporter—To confirm that MDDL is an ER localization signal it was necessary to demonstrate that this sequence could mediate the intracellular retention of a heterologous secretory reporter. For this purpose we chose VSG, which is expressed only in the bloodstream stage, obviating the need to epitope-tag this reporter for expression in procyclics. To modify VSG for secretion it was necessary to delete the C-terminal hydrophobic peptide sequence that mediates addition of the GPI membrane anchor (35). Therefore, we have engineered this gene in two forms (Fig. 1), one encoding a VSG 117 protein minus the C-terminal tail (117Δgpi) and one in which the GPI anchor sequence has been replaced with MDDL (117MDDL). Again, transformed cell lines were assayed by specific immunoprecipitation of radiolabeled reporter polypeptides (Fig. 5). Identical amounts of radiolabeled endogenous BiP were detected in all cell lines indicating that labeling conditions were equivalent for each group. Recombinant VSG polypeptides of the expected size (~59 kDa) were detected in cell extracts of both transformed cell lines (lanes 2 and 3) but not in control Pro1 cells (lane 1). An overexposure is deliberately presented for direct comparison of cell and media fractions; lower exposures of these lanes reveal single polypeptide bands of slightly different electrophoretic mobility. 117MDDL is larger (~2 kDa) than 117Δgpi because the HA9 epitope was included in the process of adding MDDL to the C terminus. The 117Δgpi reporter is exported to the culture supernatant (lane 5) in an N-terminal signal sequence-dependent manner, confirming that soluble export is possible in procyclic trypanosomes. However, as with the BiPHA9 reporter, both the low ratio of VSG secreted to VSG synthesized (compare lanes 2 and 5) and pulse/chase experiments indicate that export is inefficient. Nevertheless, attachment of the tetrapeptide, MDDL, to the C terminus of 117Δgpi abrogates the observed secretory signal (lane 6) confirming that MDDL can mediate intracellular retention of a heterologous reporter.

Secretion of the BiP ATPase Domain—Having confirmed, as predicted, that MDDL can mediate intracellular retention, we wished to examine the sequence specificity of retention more closely. We initially anticipated that some modifications of this tetrapeptide would result in partial retention phenotypes, that is, a retardation of export rather than quantitative retention. Therefore, it was necessary to develop a reporter that is exported with high efficiency so that leaky phenotypes could be detected. In addition, the slow kinetics of secretion of the BiP and VSG reporters leaves the issue of soluble secretion in trypanosomes open to considerable doubt. Failure of the VSG reporter was not so troubling since this protein is not normally expressed in procyclic trypanosomes. However, BiP is endogenous to both stages (18) necessitating some other explanation. By homology to other hsp70 proteins (36), BiP contains a ~45-kDa N-terminal ATPase core and a C-terminal peptide binding domain, and we reasoned, in the absence of a bona fide ER localization signal, that export of this reporter may be retarded by virtue of its peptide binding properties.

Therefore, we created a truncated secretory reporter (BiPN) comprised of the N-terminal ATPase domain (Fig. 1). Although still present at the C terminus of this construct, the HA9 epitope is not needed to discriminate between ~45-kDa reporter and ~72-kDa endogenous BiP since both are detected simultaneously with anti-BiP antibody, a convenient internal control. A pulse/chase experiment with a transformed cell line expressing this reporter is presented in Fig. 6A. Expression of 45-kDa BiPN reporter is very high relative to intracellular BiP protein (lane 1), which remains at constant levels throughout the chase period (lanes 1–5). In contrast, BiPN is rapidly exported from cells (lanes 1–5) into the medium (lanes 6–10). Again, as mentioned above, a defined set of high M, polypeptides are apparently associated with intracellular BiP in a transient manner (compare lanes 1 with 2–5).

To obtain an accurate estimate of the kinetics of BiPN transport, we analyzed repetitions of this experiment by densitometry (Fig. 6B). Following a short pulse, export of radiolabeled BiPN proceeds in a linear fashion for up to 2 h with a τ slightly greater than 60 min and is essentially complete in 4 h. Simultaneously, extracellular reporter increases but never reaches 100% nor does total reporter (intracellular + extracellular). This may simply reflect the difficulties inherent in such assays or may indicate that some portion of BiPN is being degraded intracellularly. Inclusion of protease inhibitors in the medium following fractionation does not increase the recovery of secreted reporter, and this issue has not been further investigated.

Analysis of ER Localization Signals—The development of the BiPN reporter convincingly demonstrates that significant soluble secretion is possible in African trypanosomes and provides the essential tool for secretory protein targeting studies. The data presented earlier strongly suggest that the C-terminal tetrapeptide, MDDL, mediates ER localization in T. brucei. We now wished to exploit the BiPN reporter to investigate the sequence specificity of this targeting signal. The BiPN gene was engineered to place various tetrapeptide sequences at the C terminus, and stably transformed cell lines were assayed for reporter export (Fig. 7). All cell lines were labeled equally as indicated by the constant amount of endogenous BiP and BiP-associated proteins detected in each cell fraction.

Attachment of several tetrapeptide sequences to the C terminus of BiPN resulted in intracellular retention of the re-
The kinetics of the basal BiPN reporter (Fig. 6) are the same and, more importantly, are essentially identical to those of the heterologous VSG reporter. These include the native BiP signal, MDDL (lanes 5 and 6), the trypanosome protein disulfide isomerase signal, KQDL (lanes 7 and 8), and the mammalian BiP sequence (37), KDEL (lanes 9 and 10). Sequences that do not mediate retention are the C-terminal tetrapeptide of the HA9 epitope, PDYA (BiPN, lanes 1 and 2), the irrelevant tetrapeptide, AVRG (lanes 3 and 4), the deletion mutant, MDD- (lanes 11 and 12), and the hybrid sequence, AVDL (lanes 13 and 14). Two secreted reporters, BiPNMDD- and BiPNAVNL, were selected for detailed analysis. Since MDD- matches the native BiP sequence in 3 of 4 positions and AVDL is a hybrid between the irrelevant AVR G and the conserved amino acids of the two active trypanosomal tetrapeptides, MDDL and KQDL, we reasoned that they may express a partial retention phenotype. Pulse/chase analyses (Fig. 8) show that the kinetics of export of these two reporters are the same and, more importantly, are essentially identical to the kinetics of the basal BiPN reporter (Fig. 6B). We conclude that these tetrapeptides are completely unable to interact with the trypanosome retention machinery.

**DISCUSSION**

We have previously cloned and characterized a homolog of the secretory hsp70 protein, BiP, in the African trypanosome, T. brucei (18). Our identification was based on overall sequence homology with BiP from other organisms and on cytolocalization studies. We demonstrate here that this protein also has a key functional property consistent with its role as a molecular chaperone, the ability to interact transiently with newly synthesized secretory proteins (33). In other systems, BiP has been shown to associate with secretory proteins such as immunoglobulin (38) and VSV-G protein (39). In bloodstream trypanosomes, we show (Fig. 2) that BiP physically associates with newly synthesized VSG and that this association is transient, disappearing rapidly as would be expected for a secretory protein with an export t1/2 of 15 min (4, 5). In addition, a discrete set of endogenous high M, polypeptides are consistently associated with BiP in both bloodstream and procyclic trypanosomes. The identities of these proteins are not known, but they are presumed to be secretory polypeptides or other chaperones involved in secretory protein folding. Whatever the nature of these proteins, the key feature of their association with BiP, as in the case of VSG, is the transience of binding.

In order to test the role of the BiP C-terminal tetrapeptide, MDDL, in mediating ER localization in trypanosomes, it was necessary to first develop soluble reporters as essential tools for secretory protein targeting studies. This was accomplished by adaptation of two known proteins of the trypanosomal secretory pathway, BiP and VSG. Secretion of VSG was induced by deletion of the C-terminal sequence that directs GPI membrane anchor addition (Fig. 5); secretion of BiP was accomplished by deletion of its C-terminal tetrapeptide, MDDL (Fig. 3). Furthermore, addition of MDDL resulted in the intracellular retention of the heterologous VSG reporter. Although the rates of export for both of these reporters were low, these results indicate in the simplest manner that MDDL is an ER localization signal.

The reason for poor export of the VSGGp reporter is not clear. Full-length VSG, when expressed in procyclic trypanosomes, is correctly GPI-anchored, dimerized, and transported to the cell surface with reasonable kinetics (t1/2 ~ 1 h),2 ruling out the simple explanation that procyclic cells are just not competent for transport of this bloodstream-specific protein. It may be that in the absence of a GPI anchor VSG cannot be correctly folded and/or dimerized and as a result is retained by ER quality control mechanisms (40). Alternatively, it is possi-
ble that GPI anchors provide some sort of positive forward transport signal. To answer this question it will be necessary to extend these results to other VSG molecules. However, it is worth noting that similar results were obtained in other systems where GPI attachment was prevented either by mutation of the GPI-addition signal (41, 42) or by inositol starvation (43).

Efficient export of BiP, on the other hand, has been observed in mammalian systems and was attributed to innate peptide binding properties that could retard transport in the absence of an ER localization signal (37). The mammalian cytoplasmic hsp70 protein, hsc70, has been biochemically characterized as having a 45-kDa N-terminal ATPase domain and a 30-kDa C-terminal peptide binding/regulatory domain (36), and this has been directly confirmed for mammalian BiP (44). We have shown (Fig. 6) that deletion of the peptide-binding domain results in the rapid and quantitative export of BiPN, providing direct experimental evidence for the conserved hsp70 domain structure in trypanosomal BiP.

The success of BiPN reporter, which is probably due to the compact globular nature of the ATPase domain (45), establishes convincingly that soluble secretion is possible in African trypanosomes. The kinetic half-time of slightly more than 60 min is the first such measurement to be made for any secretory protein, soluble or membrane-bound, in procyclic trypanosomes. Although considerably slower than the transport of membrane-bound VSG in bloodstream trypanosomes, this nevertheless seems reasonable since the doubling time of bloodstream trypanosomes (~6 h) is two to three times faster than procyclics. Bloodstream trypanosomes are also known to be substantially more active in endocytic membrane trafficking (13).

Finally, the development of effective secretory reporters has allowed us to investigate the sequence specificity of ER targeting signals in trypanosomes (Table I). Both known trypanosomal signals, MDDL and KQDL, as well as the related mammalian sequence, KDEL, mediate quantitative intracellular retention. Although the native trypanosomal signals are somewhat divergent from the canonical eukaryotic XDEL motif, the pattern that emerges is consistent with the known sequences found on native ER proteins, as well as variants that have been tested experimentally in other systems (46, 47). Trypanosomes are the most ancient eukaryotic organisms in which the sequence specificity of luminal ER protein targeting has been directly assayed; the results establish the generality of the XDEL targeting signal throughout the broad range of eukaryotic phylogenetics. Presumably, this conservation extends to the machinery that mediates ER localization as well.

Further characterization of the trypanosomal secretory pathway is required for a complete understanding of the cell biology of these important pathogenic organisms. The work we have presented, particularly the development of secretory reporters, provides the foundation for future protein targeting studies. Trypanosomes are ancient organisms and have already provided many eye-popping variations of common eukaryotic processes. It is reasonable to assume that more discoveries await, some of which may be useful in developing new strategies for control of trypanosomatid diseases.

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