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A Microsomal GTPase Is Required for Glycopeptide Export from the Mammalian Endoplasmic Reticulum*

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Bidirectional transport of proteins via the Sec61p translocon across the endoplasmic reticulum (ER) membrane is a recognized component of the ER quality control machinery. Following translocation and engagement by the luminal quality control system, misfolded and unassembled proteins are exported from the ER lumen back to the cytosol for degradation by the proteasome. Additionally, other ER contents, including oligosaccharides, oligopeptides, and glycopeptides, are efficiently exported from mammalian and yeast systems, indicating that bidirectional transport across ER membranes is a general eukaryotic phenomenon. Glycopeptide and protein export from the ER in *in vitro* systems is both ATP- and cytosol-dependent. Using a well established system to study glycopeptide export and conventional liquid chromatography, we isolated a single polypeptide species of 23 kDa from rat liver cytosol that was capable of fully supporting glycopeptide export from rat microsomes in the presence of an ATP-regenerating system. The protein was identified by mass spectrometric sequence analysis as guanylate kinase (GK), a housekeeping enzyme critical in the regulation of cellular GTP levels. We confirmed the ability of GK to substitute for complete cytosol by reconstitution of glycopeptide export from rat liver microsomes using highly purified recombinant GK from *Saccharomyces cerevisiae*. Most significantly, we found that the GK (and hence the cytosolic component) requirement was fully bypassed by low micromolar concentrations of GDP or GTP. Similarly, export was inhibited by non-hydrolyzable analogues of GDP and GTP, indicating a requirement for GTP hydrolysis. Membrane integrity was fully maintained under assay conditions, as no ER luminal proteins were released. Competence for glycopeptide export was abolished by very mild protease treatment of microsomes, indicating the presence of an essential protein on the cytosolic face of the ER membrane. These data demonstrate that export of glycopeptide export is controlled by a microsomal GTPase and is independent of cytosolic protein factors.

In eukaryotic cells, the trafficking of secretory proteins and glycoproteins is initiated upon translocation into the ER[1] lumen via the Sec61p translocon complex. During translocation, various processes, including removal of signal peptide, disulfide bond formation, and glycosylation, are initiated; and the protein enters folding pathways assisted by a number of molecular chaperones present in the ER (1, 2). As a result of these processes, a number of byproducts are generated within the ER lumen, including oligopeptides, free oligosaccharides, glycopeptides, misfolded proteins, and unassembled subunits of protein complexes (1, 3–8). Allowing these byproducts and folding intermediates into the Golgi complex or other post-ER secretory pathway compartments can lead to competition with true secretory cargo for the various modification and trafficking processes with deleterious consequences.

Eukaryotic cells have a stringent quality control machinery at the ER level that ensures that only properly folded and fully assembled proteins are allowed to exit by vesicular transport to the Golgi complex. Misfolded and unassembled proteins are retained in the ER lumen by molecular chaperones and then exported back to the cytosol, where they are processed and degraded by the ubiquitin/proteasome systems (5, 6, 9–11). Interestingly, these proteins are exported via the Sec61p translocon, the same channel through which they were imported into the ER (12, 13).

Oligosaccharides that are generated during glycoprotein synthesis by various mechanisms within the ER lumen (including the futile addition of an *N*-linked carbohydrate precursor to water instead of a protein) are exported to the cytosol for further trimming before being imported into lysosomes for complete degradation (7, 8, 14, 15). The bidirectional movement of macromolecules across the ER membrane is also an integral part of antigen presentation by MHC class I molecules of ER-targeted proteins and cytosol-derived oligopeptides. After translocation into the ER lumen via TAP, oligopeptides are sampled by the antigen presentation system, and the oligopeptides that do not fit within the MHC class I molecule active site are recycled back to the cytosol, by an unknown mechanism, for further degradation and trimming by the proteasome. Some of the resulting oligopeptides are re-imported, via TAP, into the ER lumen, where the suitable peptides assemble with MHC class I (16, 17). It was also found that ER-targeted proteins can provide a source of peptides for antigen presentation by being exported out of the ER and then subsequently degraded by the

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[1] The abbreviations used are: ER, endoplasmic reticulum; MHC, major histocompatibility complex; TAP, transporters associated with antigen processing; GDPβS, guanosine 5′-*O*-3-(thio)diphosphate; GTPγS, guanosine 5′-3-*O*-(thio)triphosphate; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; MS, mass spectrometry; MS/MS, tandem mass spectrometry; PDI, protein-disulfide isomerase; GK, guanylate kinase.

proteasome to generate peptides, which can ultimately be imported via TAP (18).

Glycopeptide export is a powerful model system for the study of retrograde ER transport *in vitro* (19, 20). The export can be achieved using heterologous sources for the membrane and cytosol, indicating the conservation of this process in eukaryotes and its essential role (20). Glycopeptide export is monitored in a cell-free system by introducing into the ER a hydrophobic iodinated tripeptide (acetyl-NYT-NH_2) that contains a canonical *N*-glycosylation sequon. Within the ER lumen, the peptide is rapidly glycosylated by oligosaccharyltransferase, thereby increasing both mass and polarity and preventing diffusion back across the ER membrane. The addition of an ATP-regenerating system (21) and cytosol and incubation at physiological temperature are required to achieve export, which is easily quantitated by capture with concanavalin A.

Recently, we reported that this system is closely related to protein export based on biochemical criteria allowing the differentiation of the retrograde transport of glycopeptides from the export of free oligosaccharides, hence suggesting that this system could allow identification of factors common to both peptide and protein retrotranslocation mechanisms (22). As glycopeptide export from rat liver microsomes is dependent upon cytosol, in common with protein export *in vitro* systems, we exploited this system to identify the cytosolic protein(s) required for retrograde translocation. Surprisingly, we were able to demonstrate that GTP can substitute for cytosol; and hence, glycopeptide export from mammalian microsomes does not require cytosolic proteins.

EXPERIMENTAL PROCEDURES

Materials

Acetyl-NYT-NH_2 was synthesized by Albachem Ltd. ATP, GDP-mannose, GDP, GMP, cGMP, GDPβS, GTPγS, and *Canavilia* lectin (concanavalin A)-Sepharose were from Sigma. ^{125}I (100 mCi/ml) was from Amersham Pharmacia Biotech. Rat livers were from Harlan Sera Lab Ltd. (Loughborough, UK). Creatine kinase and creatine phosphate were from Roche Molecular Biochemicals. Other chemicals were obtained from commercial sources and were of the highest purity available.

Peptide Iodination and Glycopeptide Export Assay of Rat Liver Microsomes

The acetyl-NYT-NH_2 peptide was iodinated with ^{125}I using chloramine T as described by Wieland *et al.* (23). The iodinated peptide was purified from unincorporated ^{125}I by binding to a Sep-Pak C_{18} light cartridge and then eluting with 60% acetonitrile in 0.1% trifluoroacetic acid. The iodinated peptide was used within 1 month of iodination.

Glycopeptide Export Assay of Rat Liver Microsomes

The glycopeptide export assay was performed exactly as described by Romisch and Ali (20) as follows.

Loading—Crude rat liver membranes (100 μl; $A_{280} \sim 200$) were suspended in 1 ml of ice-cold B88 buffer (20 mM HEPES-KOH (pH 7.4) 150 mM KOAc, 250 mM sorbitol, and 5 mM $Mg(OAc)_2$) containing 0.5 M KCl or NaCl and placed on a rotator for 15 min at 4 °C. The membranes were then sedimented in a cooled (4 °C) Eppendorf microcentrifuge at 20,000 × *g* for 5 min and washed with 1 ml of B88 buffer (pH 7.4). The membranes were resuspended in B88 buffer (200 μl); ^{125}I-acetyl-NYT-NH_2 was added at 1×10^7 cpm/100 μl; and the reaction mixture was incubated at 10 °C for 20 min. This allowed the peptide to enter the ER membranes and to be glycosylated by the endogenous oligosaccharyltransferase. To remove the unglycosylated tripeptide, 2 × 1 ml of ice-cold B88 buffer was added, and the membranes were then sedimented as described above and resuspended in 200 μl of B88 buffer.

Export—Loaded membranes (5 μl of washed membranes; $A_{280} \sim 10$) were placed in Eppendorf tubes on a precooled ice-cold block, and the various components (as necessary) were added to individual tubes in the following order. B88 buffer was added to bring the final volume to 25 μl; 2.5 μl of partially purified rat liver cytosol (5 μg of protein) was then added; and 2.5 μl of a 10× ATP-regenerating system was added last and mixed quickly (final concentrations: 1 mM ATP, 40 mM creatine phosphate, 0.2 mg/ml creatine kinase, and 50 μM GDP-mannose). Export reactions were initiated by incubating the tubes at 32 °C for a specified period of time, after which the membranes were quickly sedimented at 4 °C in microcentrifuge, and the supernatant (cytosol) was separated from the pellet (membranes). Very little ongoing glycosylation was observed during the export stage of the assay as evidenced by the constant amount of total glycopeptide recovered in both the supernatant and membrane fractions and by the observation that at least 90% of the total iodinated peptides recovered after the loading and washing steps were glycosylated (22).

Quantitation of Glycopeptide Export—To each supernatant or pellet fraction was added 100 μl of 2% SDS, and samples were heated immediately at 95 °C for 5 min. The samples were cooled, and 1 ml of concanavalin A buffer (20 mM Tris-HCl (pH 7.5), 0.5 M NaCl, and 1% Triton X-100) was added, followed by 50 μl of 50% immobilized concanavalin A-Sepharose slurry; and the mixture was placed on a rotator at room temperature for 2 h. The beads were sedimented using a microcentrifuge and washed once (by suspending and then sedimenting by brief centrifugation) with each of the following: immunoprecipitation buffer (15 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, and 0.1% SDS), urea buffer (100 mM Tris-HCl (pH 7.5), 200 mM NaCl, 1% Triton X-100, and 2 M urea), and concanavalin A buffer. Radioactivity was measured by γ-counting using an LKB 1275 mini-γ-counter. Glycopeptide export was determined as the percentage of radioactivity (counts/min) in the supernatant relative to the total counts in both the supernatant and membranes.

Preparation of Rat Liver Microsomes and Purification of the Glycopeptide Export Factor

Rat liver rough membranes and cytosol were prepared as described previously (20). Cytosol was prepared from frozen rat livers purchased from Harlan Sera Lab Ltd., and rough microsomes from fresh rat livers were obtained from the CBS unit of the Imperial College. Glycopeptide export activity (obtained from a 100,000 × *g* cytosolic fraction of 18 rat livers) was used for the purification of the glycopeptide export factor as follows. The clarified cytosol (300 ml) was subjected to ammonium sulfate precipitation, and glycopeptide export activity was precipitated between 40 and 60% ammonium sulfate. The protein pellet was dissolved in 100 ml of 20 mM Tris-HCl (pH 8.0) containing 1 mM DTT and then dialyzed against the same buffer for 16 h. Insoluble material was removed by centrifugation at 15,000 × *g*, and the supernatant was loaded onto a Q-Sepharose fast flow column (5 × 20 cm) at 5 ml/min. After washing the column extensively (1500 ml) with the same buffer, the bound proteins were eluted with a KCl gradient (0–1.0 M) over 1000 ml in the same buffer. Fractions (10 ml each) were collected and assayed for glycopeptide export activity, which showed that the active proteins eluted between 300 and 500 mM KCl. The active fractions were pooled (160 ml), and their protein contents were precipitated with 70% ammonium sulfate. The precipitated proteins were dissolved in 40 ml of 25 mM Tris-HCl (pH 7.0) containing 1 mM DTT and then loaded in four portions (10 ml each) onto a Superdex G-75 26/60 HiLoad column. Elution was in the same buffer at 2 ml/min. Fractions (5 ml each) were collected and assayed for glycopeptide export activity, which was recovered in fractions 36–44. The fractions from four runs were pooled (150 ml) and then added to 50 ml of hydroxylapatite type I (Sigma) slurry in the above buffer and mixed gently for 30 min. The hydroxylapatite was removed by centrifugation, and the supernatant was further clarified by filtering thorough a glass-fiber filter. Most of the activity was recovered in the unbound fraction, brought to 1.5 M ammonium sulfate, and then loaded onto a 5-ml phenyl-Sepharose fast flow column pre-equilibrated with the same buffer. Bound proteins were eluted with a decreasing ammonium sulfate gradient (1.5 to 0 M). Fractions (2 ml each) were collected, and the active protein was found in fractions 28–36 (0.6 to 0.3 M ammonium sulfate). Active fractions (~20 ml) were pooled and ultra-filtered through a 10-kDa cutoff membrane, and the buffer was exchanged with 20 mM Tris-HCl (pH 8.0) containing 1 mM DTT and then chromatographed on a Mono Q fast protein liquid chromatography column. After loading the sample onto Mono Q, the column was washed with 5 ml of loading buffer, followed by a KCl gradient (0–0.5 M) over 20 ml. Fractions (1 ml each) were collected from the wash and gradient and assayed for glycopeptide export activity, which was recovered in fractions 13 and 14 (200–220 mM KCl). SDS-PAGE analysis and silver staining indicated the presence of two protein bands in fraction 14, one of which was present in the inactive fraction (fraction 12), leaving the one at ~23 kDa as the likely active protein.

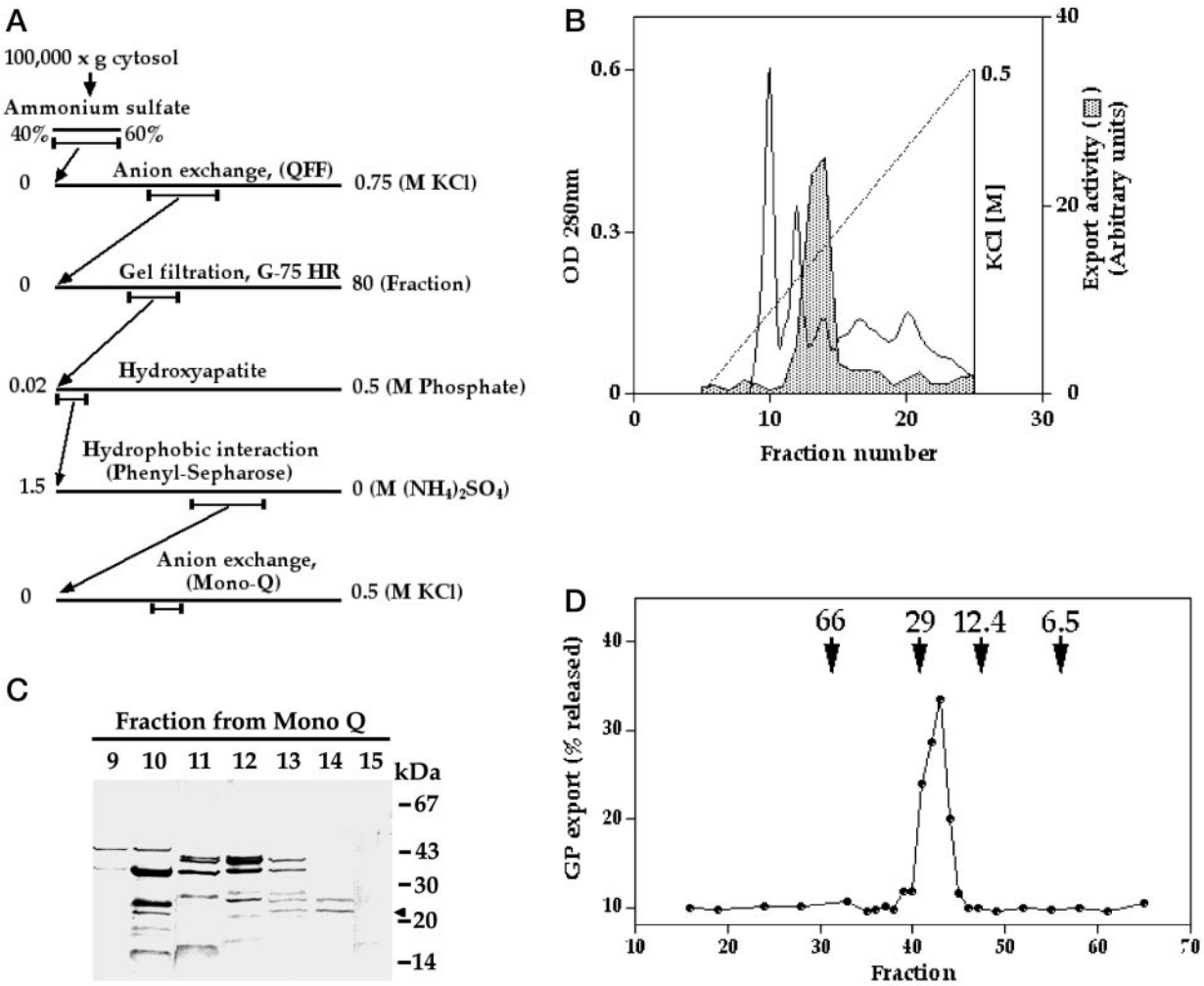

FIG. 1. **Purification of the glycopeptide export factor from rat cytosol.** *A*, schematic representation of the purification. The active fractions from each step (represented by a *bar*) were pooled and loaded onto the next chromatography column as described under "Experimental Procedures." The start of the gradient (or fractions) for each chromatography step is shown on the left, and the end is shown on the right. *QFF*, Q-Sepharose fast flow. *B*, elution profile of the final chromatography step of the purification (Mono Q HR5/5 fast protein liquid chromatography). After loading, the column was washed with 5 ml of buffer, followed by a KCl gradient (0–0.5 M) over 20 ml. 1-ml fractions were collected and assayed for glycopeptide export activity (*shaded*) and protein content (A_{280}). Only fractions 13 and 14 were active for glycopeptide export. *C*, SDS-PAGE analysis of proteins present in fractions 9–15 of the Mono Q column. 15 μl from each fraction was run on 15% SDS-polyacrylamide gel under denaturing conditions and then silver-stained. Fractions 13 and 14 contained one unique protein at ~23 kDa (*arrowhead*) that was not present in fractions 12 and 15, which are inactive for glycopeptide export. Fraction 15 was electrophoresed on the same gel as the other fractions, but after the molecular standards lane, and therefore was manipulated with Adobe Photoshop to place it adjacent to fraction 14 for presentation. *D*, the glycopeptide (*GP*) export factor migrates as a 23-kDa protein on a gel filtration column. 2 ml of the 40–60% ammonium sulfate-precipitated fraction, which contained the glycopeptide activity, was loaded onto a Superdex G-75 26/60 HiLoad column pre-equilibrated with 25 mM Tris-HCl (pH 7.5) containing 100 mM NaCl and 1 mM DTT and then eluted in the same buffer at 30 ml/h. 5-ml fractions were collected, and 2 μl from selected fractions was used in the glycopeptide export assay. Protein gel filtration standards (Sigma MW-GF-70 kit) were also chromatographed under the same conditions, and their elution positions (in kilodaltons) are indicated by *arrows*. The active glycopeptide protein eluted at ~23 kDa. Protein standards were bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), cytochrome *c* (12.4 kDa), and aprotinin (6.5 kDa).

Mass Spectrometry

Peptide sequence data were obtained by in-gel digestion with trypsin and analysis of the products by both matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MS) and liquid chromatography electrospray ionization MS/MS using a quadrupole ion-trap mass spectrometer as described (24). Peptide sequences were used to identify the protein by searching the NCBI Nonredundant Database with the programs PROFOUND and PEPFRAG (25).

Assessment of Microsomal Latency

After incubating the glycopeptide export reactions at the specified temperatures and the specified period of times, tubes were cooled on ice; 25 μl of ice-cold B88 buffer was added; and the reactions were spun down at 20,000 $\times$ *g* for 10 min at 2 °C. The supernatant (50 μl) was removed, and the membrane pellet was resuspended in 50 μl of B88 buffer. SDS was added from a stock solution to a final concentration of 2% to both fractions and heated at 95 °C for 4 min. 15 μl of each fraction (supernatant and pellet) was removed to a new tube for protein-disulfide isomerase (PDI) analysis, and the rest was used for glycopeptide quantitation as described above. SDS sample buffer was added to the samples designated for PDI analysis, heated for an additional 3 min, and then electrophoresed on 12.5% SDS-polyacrylamide gels. As a control, Triton X-100 was used for the total release of ER luminal contents by membrane solubilization prior to fractionation into soluble and membrane-associated fractions. Proteins were transferred from the gels to Hybond™ ECL nitrocellulose membranes (Amersham Pharmacia Biotech) and probed with anti-PDI polyclonal antibodies. Peroxidase (Sigma)-conjugated secondary antibodies were used along with a chemiluminescence detection kit to visualize anti-PDI antibodies.

RESULTS

Purification of a Cytosolic Component That Supports Glycopeptide Export—A fully active glycopeptide export fraction was purified from rat liver by ammonium sulfate precipitation, followed by sequential chromatography on Q-Sepharose ion exchange, Superdex G-75 gel filtration, hydroxylapatite, phe-

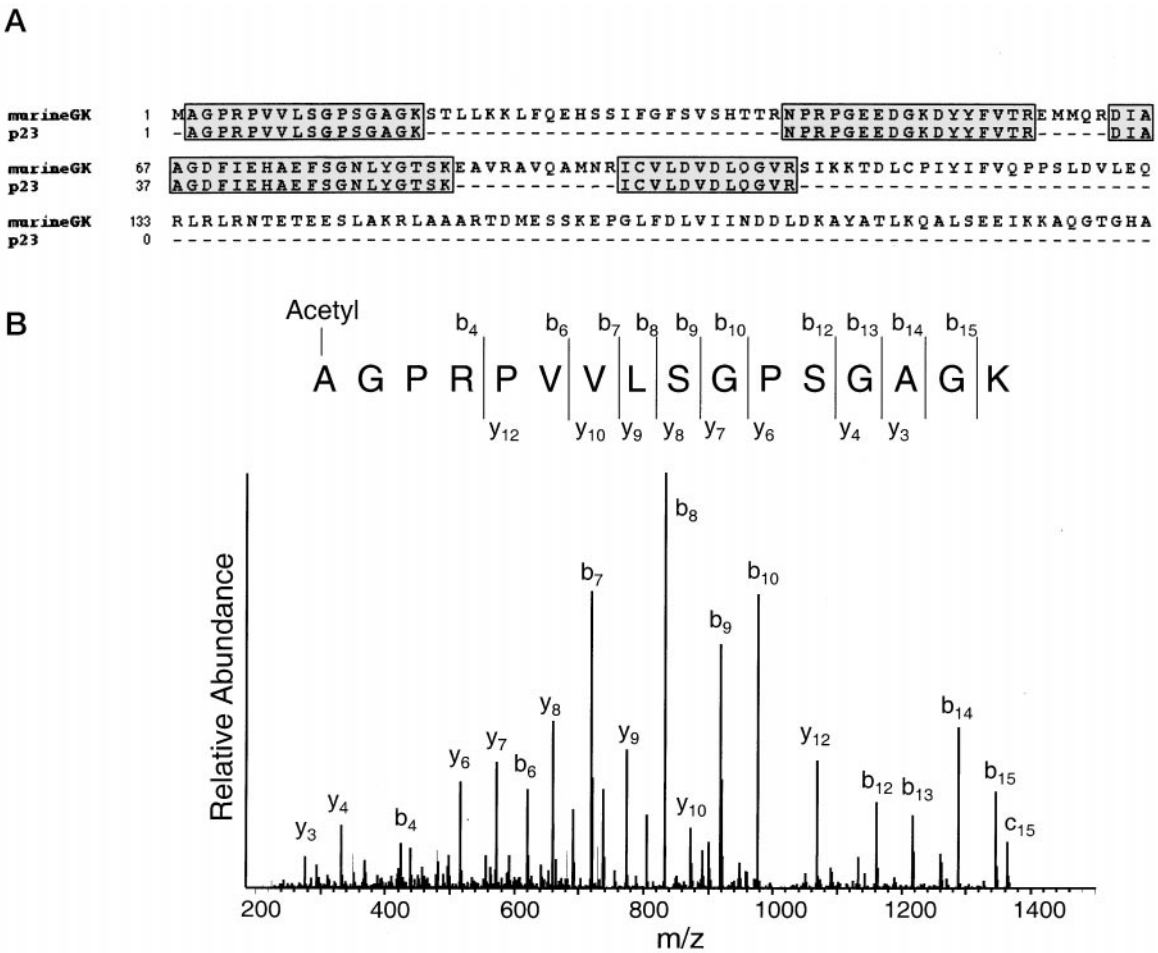

FIG. 2. **Identification of the active protein for glycopeptide export as guanylate kinase.** *A*, peptide sequences determined from MS data are shown aligned with the sequence of mouse GK. *B*, liquid chromatography electrospray ionization MS/MS of a tryptic peptide from the 23-kDa protein band. The molecular mass of the peptide was determined to be 1490.83 Da, which is close to that calculated for the amino-terminally acetylated peptide shown (1490.82 Da). The fragmentation nomenclature is as described previously (37).

nyl-Sepharose hydrophobic interaction, and Mono Q ion exchange columns (Fig. 1*A*). From 7 g of total soluble hepatic proteins, we purified ~50 μg of the active export factor. The elution profile of total protein (A_{280}) and glycopeptide export activity from the final Mono Q chromatography step is shown in Fig. 1*B*. The activity eluted at ~200 mM KCl and was collected as two fractions (fractions 13 and 14) coinciding with an A_{280} peak. Proteins from these and adjacent fractions were analyzed by SDS-PAGE, followed by silver staining (Fig. 1*C*). The active fractions contained a single common unique protein band with a molecular mass of 23 kDa (*arrowhead*); fraction 14 contained two proteins, one at 23 kDa and a larger one at 27 kDa. This latter protein was also present in fraction 12; and as this fraction was devoid of export activity, we concluded that the export activity was most likely to be the 23-kDa protein, which we designated p23. To confirm this assignment, we used partially purified cytosol, corresponding to the active fractions from the first ion exchange chromatography step of the purification, and resolved the export activity by gel filtration on a Superdex G-75 high resolution gel permeation column in 20 mM Tris-HCl (pH 7.5) containing 1 mM DTT and 150 mM NaCl. By comparison with standard proteins, the activity eluted as a tight peak at ~22.5 kDa (Fig. 1*D*). Because both the denatured and native proteins have an identical apparent molecular mass, the export factor is likely to be globular, soluble, and monomeric, typical of a cytosolic protein.

Identification of p23 as Guanylate Kinase—To identify p23, we further purified ~25 μg of the export factor by preparative SDS-PAGE. The protein in the 23-kDa band was subjected to in-gel digestion with trypsin, yielding a mixture of proteolysis products that was analyzed by both matrix-assisted laser desorption/ionization time-of-flight MS and liquid chromatography electrospray ionization MS/MS using a quadrupole ion-trap mass spectrometer (24). Data obtained from the resulting mass spectra were used to identify the protein by searching the NCBI Nonredundant Database with the programs PROFOUND and PEPFRAG (25). Liquid chromatography electrospray ionization MS/MS revealed the presence of four peptides with sequences identical to portions of the known sequence of mouse guanylate kinase (Fig. 2*A*). The MS/MS spectrum of one of these peptides is shown in Fig. 2*B*, which shows that this spectrum corresponds to the amino-terminal peptide from which the initiator methionine has been removed and in which the alanine at position 2 has been acetylated. The MS analysis identified fully 44% of the guanylate kinase sequence, providing unambiguous identification of the protein as rat guanylate kinase (GK) (GMP kinase; EC 2.7.4.8). Identification of GK was unexpected, as this activity was not predicted to be involved in ER-to-cytosol export (26). GK catalyzes the interconversion of GMP and GDP in the following equilibrium: GMP + ATP $\leftrightarrow$ GDP + ADP. It is therefore a critical enzyme for dGTP and GTP biosynthesis and hence might be involved in guanine nucleotide-mediated signal transduction pathways (27). Therefore, we sought to test the essential role of GK in the export system by several criteria.

First, many nucleotide-binding enzymes, including GK, bind

tightly to dye affinity matrices via the nucleotide-binding site and can be eluted specifically with nucleotides. We analyzed the interaction of partially purified glycopeptide export factor by Cibacron blue-Sepharose chromatography. Greater than 70% of the glycopeptide export activity bound to the affinity matrix and was eluted with 1 mM ATP (Fig. 3*A*), consistent with both the properties of GK and the prediction of a nucleotide-binding site in the active factor. GK behaved in a similar fashion, under the conditions used in this study, on a Cibacron blue-Sepharose column, which was a crucial step in the original purification to homogeneity from bovine tissue (28). Second, partially purified *Sus scrofa* brain cytosolic GK also promoted glycopeptide export from rat liver microsomes in the presence of ATP and an ATP-regenerating system under standard assay conditions (data not shown). Although it is unlikely that this source would also contain a copurifying ER export factor, we cannot rule this out based on these data alone. Hence, third, highly purified recombinant *Saccharomyces cerevisiae* guanylate kinase expressed in *Escherichia coli* (29) was assayed and substituted fully for cytosol in the glycopeptide export system when added with ATP and an ATP-regenerating system (Fig. 3*B*). Recombinant yeast GK elicited glycopeptide export at the same level as partially purified rat liver cytosolic factor (Fig. 3*B*, *lanes 5–7 versus lane 3*). GK, when added alone, had no effect on export and hence did not compromise membrane integrity (Fig. 3*B*, *lane 4*). These data indicate that GK is functionally capable of supporting ER export, and the reconstitution with GK from two totally independent sources effectively rules out the presence of an undetectable moiety in our preparation as the true export factor.

GTP and ATP Alone Support Microsomal Glycopeptide Export—The data above strongly suggest involvement of guanine nucleotides in the control of ER export. In common with most other *in vitro* transport systems, our assay contains an ATP-regenerating system to provide a constant supply of nucleotide triphosphate (21). This system is also capable of generating GTP from GDP; and hence, it is likely that the export system requires GTP, indicating that the true role of GK in this system would be to recycle guanine nucleotides into the regenerating system. We directly tested this possibility by attempting to support export by adding nucleotides in the absence of any cytosolic proteins. GMP and cGMP at up to 1 mM failed to support glycopeptide export when added together with ATP (data not shown). By contrast, GDP and GTP substituted fully for the cytosolic factor (Fig. 4, *A* and *B*, respectively). In the presence of ATP and an ATP-regenerating system, 1 μM GDP or GTP was sufficient to bypass the requirement for cytosol, and maximal transport was achieved at 1–5 μM nucleotide. However, neither GDP nor GTP could support export in the absence of ATP. This result demonstrated a requirement for both nucleotides and importantly ruled out the possibility that the guanine nucleotides were impure and providing a source of ATP rather than being directly required. GDP supports export because it can be converted to GTP by the regeneration system, whereas GMP, in the absence of GK, cannot be converted to GTP. This interpretation is supported by the observation that non-hydrolyzable analogues of GDP and GTP (GDPβS and GTPγS, respectively) inhibited export, unambiguously confirming an absolute requirement for nucleotide hydrolysis (Fig. 4, *A* and *B*).

To demonstrate that GTP is the required component and to rule out the possibility that cytosol has an additional role, we compared the kinetics of export using cytosol and guanine nucleotides. A time course of glycopeptide export in the absence or presence of 10 μM GDP is shown in Fig. 4*C*. Export reactions with 10 μM GTP were very similar (data not shown). Export in the presence of guanine nucleotide was more rapid than under other

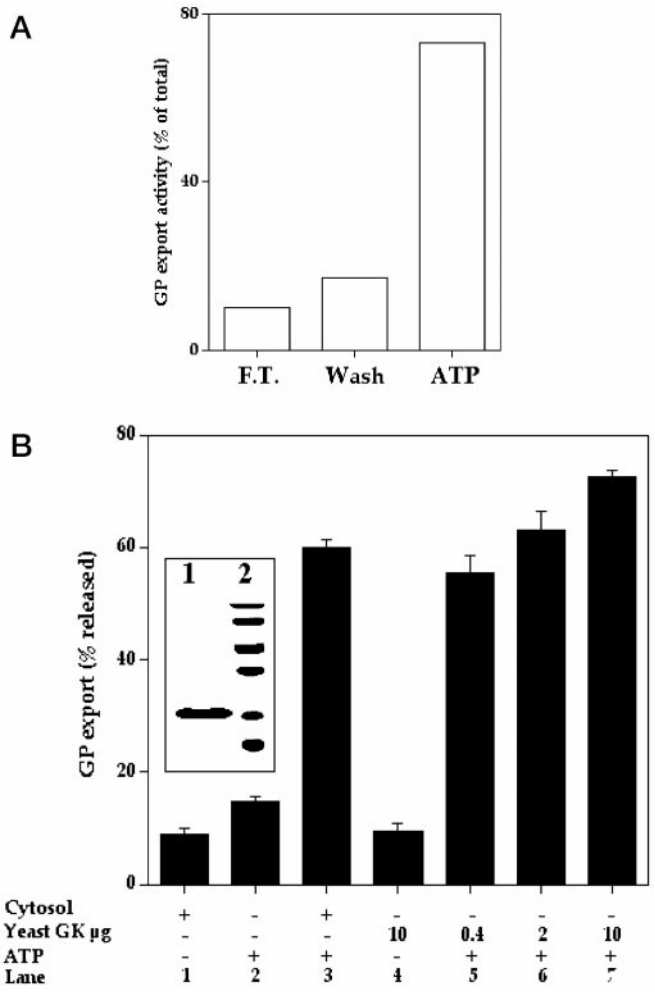

FIG. 3. **Reconstitution of glycopeptide export with guanylate kinase.** *A*, the glycopeptide (*GP*) export cytosolic factor binds to Cibacron blue-Sepharose. 1 ml of partially purified export factor (after gel filtration) was dialyzed against 5 mM HEPES-KOH (pH 7.0) and loaded onto a 1-ml Cibacron blue-Sepharose HiTrap column (Amersham Pharmacia Biotech) at 0.25 ml/min. The column was washed with 2 ml of loading buffer containing 50 mM NaCl and 1 mM $MgCl_2$. The bound material was eluted with loading buffer plus 1 mM ATP. Fractions (0.5 ml each) were collected throughout the chromatography and assayed for glycopeptide export activity. Total activity in the flow-through (*F.T.*), wash, and eluted fractions was determined and shows that ~75% of the activity was retained by the Cibacron blue-Sepharose and could be eluted with ATP. *B*, recombinant yeast guanylate kinase substitutes for rat liver cytosol in glycopeptide export from rat liver microsomes. Purified wild-type *S. cerevisiae* GK expressed in *E. coli* (29) was added to glycopeptide-loaded microsomes in place of rat cytosol as described under "Experimental Procedures." *Inset*, demonstration of purity of recombinant yeast guanylate kinase by Coomassie staining of the SDS-polyacrylamide gel. *Lane 1*, ~10 μg of yeast GK; *lane 2*, Amersham Pharmacia Biotech molecular mass standards.

conditions. In full reactions containing ATP and cytosol (*open circles*), but without guanine nucleotide, the time required for export of 50% of the total loaded peptide was ~11 min, whereas in the presence of 10 μM GDP (*closed circles*), the corresponding period was ~2.5 min. The measured time required for 50% export under standard assay conditions in the presence of ATP, cytosol, and 10 μM GTP was ~2.3 min (data not shown). The complete absence of additive export activity when cytosol was added to GTP-containing export reactions effectively rules out a role for additional cytosolic factors. The more rapid export with GDP/GTP compared with cytosol alone is most likely due to increased local GTP concentrations achieved by supplementing reactions with the guanine nucleotide rather than by generating GTP

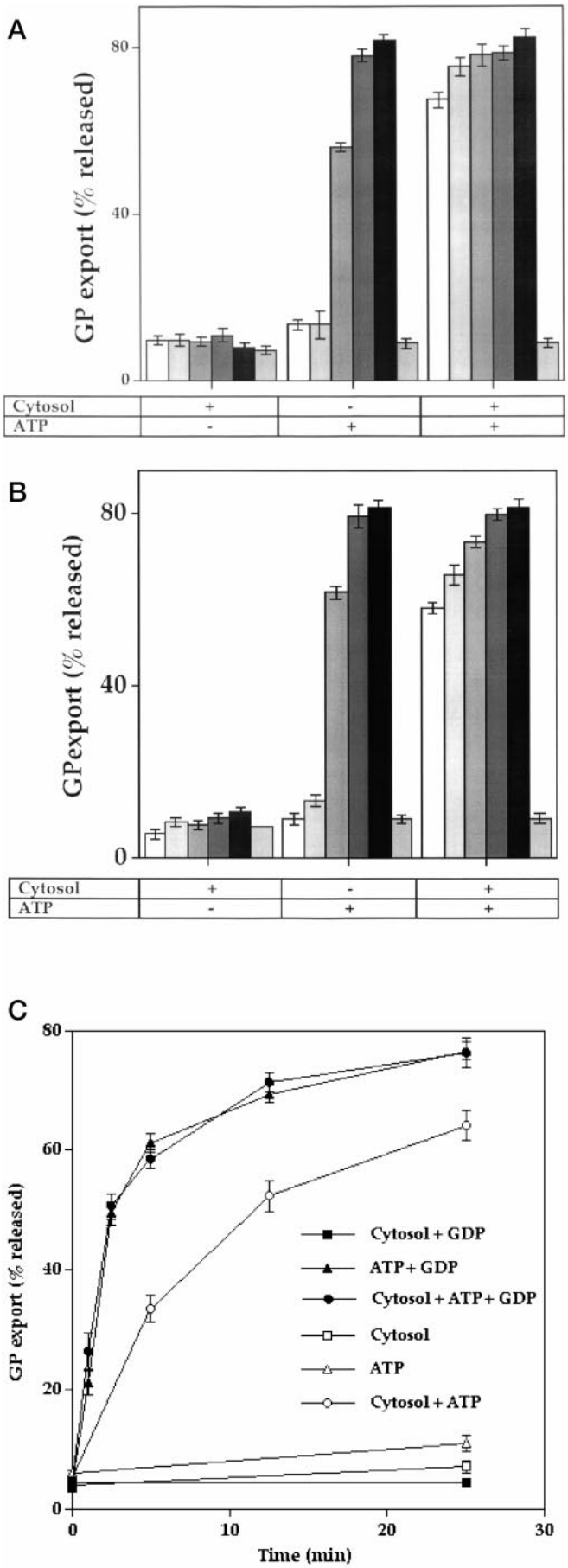

FIG. 4. **GTP hydrolysis is required for glycopeptide export from rat liver microsomes.** *A*, glycopeptide (*GP*) export assays were carried out in the absence of GDP (*white bars*) or in the presence of 0.25, 1, 5, and 25 μM GDP (*light-gray* to *black bars*) or 100 μM GDPβS (*far right bars*). All reactions were performed at 32 °C for 15 min in duplicate in the presence of cytosol, ATP, or both as indicated. *B*, glycopeptide export assays were carried out in the absence of GTP (*white bars*) or in the presence of 0.25, 1, 5, and 25 μM GTP (*light-gray* to *black bars*) or 100 μM GTPγS (*striped bars*). Conditions were as described for *A*. *C*, shown is a time course of glycopeptide export from rat liver microsomes in the absence (*open symbols*) or presence (*closed symbols*) of 10 μM GDP. Glycopeptide-loaded microsomes were incubated in the presence of cytosol only (*squares*), ATP only (*triangles*), or both cytosol and ATP (*circles*). The assays were carried out in duplicate at 32 °C.

indirectly via GK. Overall, the data above indicate that glycopeptide may be exported from rat liver microsomes in the absence of cytosolic factors. GK is able to supply hydrolyzable guanine nucleotide to the system, but the presence of the enzyme *per se* is not a requirement.

The Source of Guanine Nucleotide in the Assay System Is GDP-mannose—The apparent dependence of glycopeptide export on GK indicated the presence of a guanine nucleotide source and specifically GMP. Specifically, both GDP and GTP can substitute for cytosol/GK, but GMP cannot within the assay system. Hence, we reasoned that GK is required to convert GMP to GDP. GDP is then converted to GTP by the ATP-regenerating system. A clear source of guanine nucleotide in the assay is GDP-mannose, present at 50 μM as part of the original ATP-regenerating system formulation (21). To test if GDP-Man is a source of GMP, we performed export assays in the presence or absence of GDP-Man. The nucleotide sugar was not essential for maximal glycosylation of the peptide, consistent with the large quantity of pre-existing dolichol-linked oligosaccharide donor present in mammalian microsomes (data not shown). When GDP-Man was omitted from the ATP-regenerating system (Fig. 5, *ATP**), no glycopeptide export above background was detected in the presence of either cytosol or GK (*lanes 5* and *7*). However, export was restored by the addition of GMP (Fig. 5, *lanes 8* and *9*) or GTP (data not shown), demonstrating that GDP-Man is a source of GMP.

Maintenance of Luminal Latency—An additional concern with the ability of GTP to reconstitute glycopeptide export was the possibility that latency of luminal components may be compromised. The fusion of microsomes to produce networks and large structures has been reported on several occasions (30), including an increased permeability to low molecular mass compounds, specifically monosaccharides (31). The importance of this fusion behavior to normal ER function is not known; but to exclude this phenomenon as accounting for the GTP-mediated glycopeptide export detected here, we analyzed soluble and pellet fractions in an export assay for release of a luminal marker protein, PDI. No significant release of PDI was observed under the conditions used for glycopeptide export. Incubation (32 °C, 10 min) of glycopeptide-loaded microsomes with the ATP-regenerating system and either partially purified rat liver export fraction or recombinant yeast GK resulted in ~50% export of glycopeptides (Fig. 6*A*, *reactions 5* and *8*). On the other hand, PDI was predominantly present in the membrane fractions, and no significant release was detected under these conditions (Fig. 6*B*, *reactions 5* and *8*). Glycopeptide and PDI localization was determined using the same reactions (see legend to Fig. 6). Control reactions showed only background release of glycopeptide and no release of PDI (Fig. 6, *A* and *B*, *reactions 1–4*, *6*, and *7*). We also confirmed that GTP at low micromolar concentrations (1 and 5 μM) had no effect on PDI compartmentalization (Fig. 6, *C* and *D*). No detectable amounts of PDI were released into the incubation supernatants under the conditions where ~70% glycopeptide was exported (Fig. 6, *C* and *D*, compare *reactions 3* (for 1 μM GTP) and *6* (for 5 μM

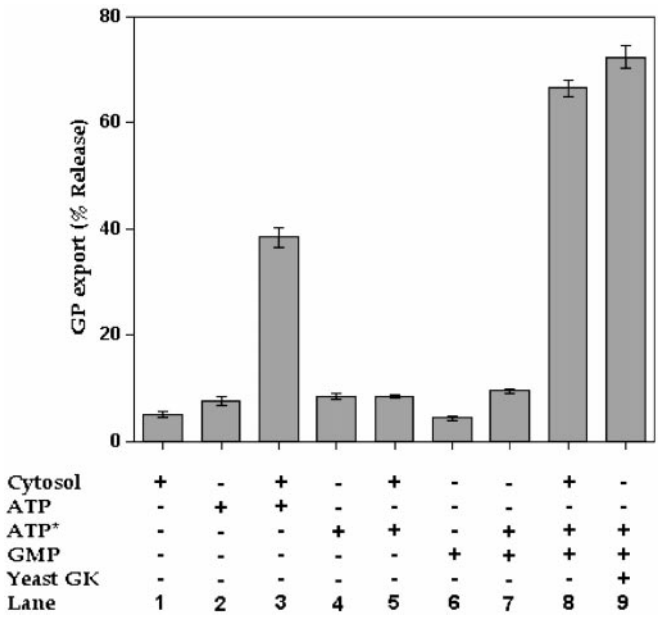

FIG. 5. **Guanine nucleotide in the assay is derived from GDP-mannose.** Glycopeptide (*GP*) export from mammalian microsomes was performed using either the regular ATP-regenerating system (*ATP*; see "Experimental Procedures" for detailed composition) or the same system but omitting GDP-man (*ATP**). The export reactions were performed at 32 °C for 10 min. Adding rat liver cytosol and a regular ATP-regenerating system (*lane 3*) resulted in 40–50% release. However, when the GDP-Man-free ATP-regenerating system was used, no export was observed (*lane 5*). Adding 10 μM GMP to this reaction restored export (*lane 8*). Purified yeast GK substituted fully for rat liver cytosol (*lane 9*). Control reactions (GMP or ATP* alone or their combination) had no effect (*lanes 4*, *6*, and *7*, respectively).

GTP)). When Triton X-100 was added to the reactions prior to fractionation into cytosol and membranes, PDI was quantitatively released into the soluble fraction (Fig. 6*E*). We conclude that under the conditions that allowed the export of glycopeptides, the ER luminal soluble contents were not released, indicating that the integrity of these membranes was not compromised, confirming the physical relevance of our observations.

Requirement for a Microsomal Cytosolically Oriented Protein in Glycopeptide Export—The microsomes used in the assay were routinely stripped of peripheral membrane proteins with 0.5 M KCl or NaCl. Therefore, we suspected an integral membrane GTPase as being the regulator of export activity and hence the site of GTP requirement. To test this hypothesis, we used a method developed for analysis of the signal recognition particle receptor and treated microsomes with very small amounts of trypsin to selectively inactivate cytosolically oriented proteins before assaying for export activity (32). Treatment with >1 μg/ml trypsin for 10 min on ice prior to loading with the iodinated tripeptide was sufficient to abolish export activity (Fig. 7), whereas protease treatment under these conditions did not affect peptide import or subsequent glycosylation (data not shown). SDS-PAGE analysis indicated that several protein species were released, but Western blot analysis of the microsomes indicated that the signal recognition particle receptor α subunit was still present on the microsomes even following exposure to trypsin treatment that fully inactivated glycopeptide export competence (data not shown). Hence, a component of the glycopeptide export system is exquisitely sensitive to protease.

DISCUSSION

There is increasing evidence that movement of macromolecules across ER membranes is bidirectional and considerably more complex than appreciated as recent as 5 years ago. At least four classes of byproduct of the ER synthetic machinery can be re-exported from the ER lumen into the cytosol: proteins, peptides, glycopeptides, and oligosaccharides. In addition, maintaining the relative oxidative environment of the ER lumen, an important property for protein folding, requires translocation of both GSH and GSSG across the ER membranes in both directions (33). Biochemically, there are clear distinctions between the export reactions for each of these classes, which implies the involvement of a large number of protein factors and possibly several distinct routes out of the ER. In addition, some of the exported molecules may require cytosolic factors for their export, in particular Hsp90, recently implicated in the degradation of mutant forms of the insulin receptor (34). This system is also an important part of the immunological surveillance mechanism for sampling of peptides for presentation to MHC class I, as oligopeptides are known to be exported from the ER for processing by the proteasome prior to reentry through the TAP transporter for interaction with nascent class I molecules (5, 16, 17).

The Sec61p translocon complex has been implicated in the export and degradation of misfolded proteins, but the exact mechanisms for these processes remain largely undefined (12). This pathway is an important component of the quality control system, with the major function of delivering proteins to the ubiquitin/proteasome degradation machinery. Oligosaccharide export is also most likely a quality control process and functions to remove glycans that have been generated from dolichol-linked precursors transferred to water rather than to a polypeptide chain. Important features of this system are a requirement for luminal calcium, deglucosylation of the glycan, and sensitivity to mannosides, suggesting involvement of an ER luminal lectin (7, 8, 35). By contrast, glycopeptide export is unaffected by manipulation of ER calcium levels, is not affected by mannosides, and does not require removal of glucose residues, consistent with the hypothesis that the calnexin system is not involved in the export of this class of substrate (22). However, both oligosaccharide and glycopeptide retranslocation systems require ATP, whereas the translocon itself has not been identified for these substrates.

The observations in this study demonstrate that glycopeptide export does not require cytosolic protein factors. We have been able to reconstitute efficient glycopeptide release by the addition of either GDP or GTP. However, we take the ability of GDP to bypass the cytosol requirement to be due to efficient conversion to GTP by the ATP-regenerating system included in the assay. Guanine nucleotide is provided by GDP-Man present in the ATP-regenerating system (21). The guanine nucleotide cannot be GDP generated directly from GDP-Man by glycosyltransferase activities on the cytosolic face of the ER producing dolichol-P-Man and lipid-linked oligosaccharides because the ATP-regenerating system cannot support export unless GTP, GDP, or, more importantly, GK is added. The ability of GK to bypass the GDP/GTP requirement strongly indicates that GMP is generated. Our data indicate that GDP-Man or GMP can support export in the context of an ATP-regenerating system. This is analogous to the situation in *S. cerevisiae*, where GDP-Man is imported into Golgi membranes for mannosylation of *N*- and *O*-linked glycans. The GDP product is dephosphorylated to GMP by Golgi luminal guanosine diphosphatase before re-export back to the cytosol (36).

Most significantly, non-hydrolyzable GTP analogues do not support export. Hence, we conclude that GTP hydrolysis is required; and thus, a GTPase activity is involved in regulating glycopeptide export. Clearly, this suggests that a GTPase is important in the control of the translocation system and potentially may serve to gate the translocon itself. Most significantly,

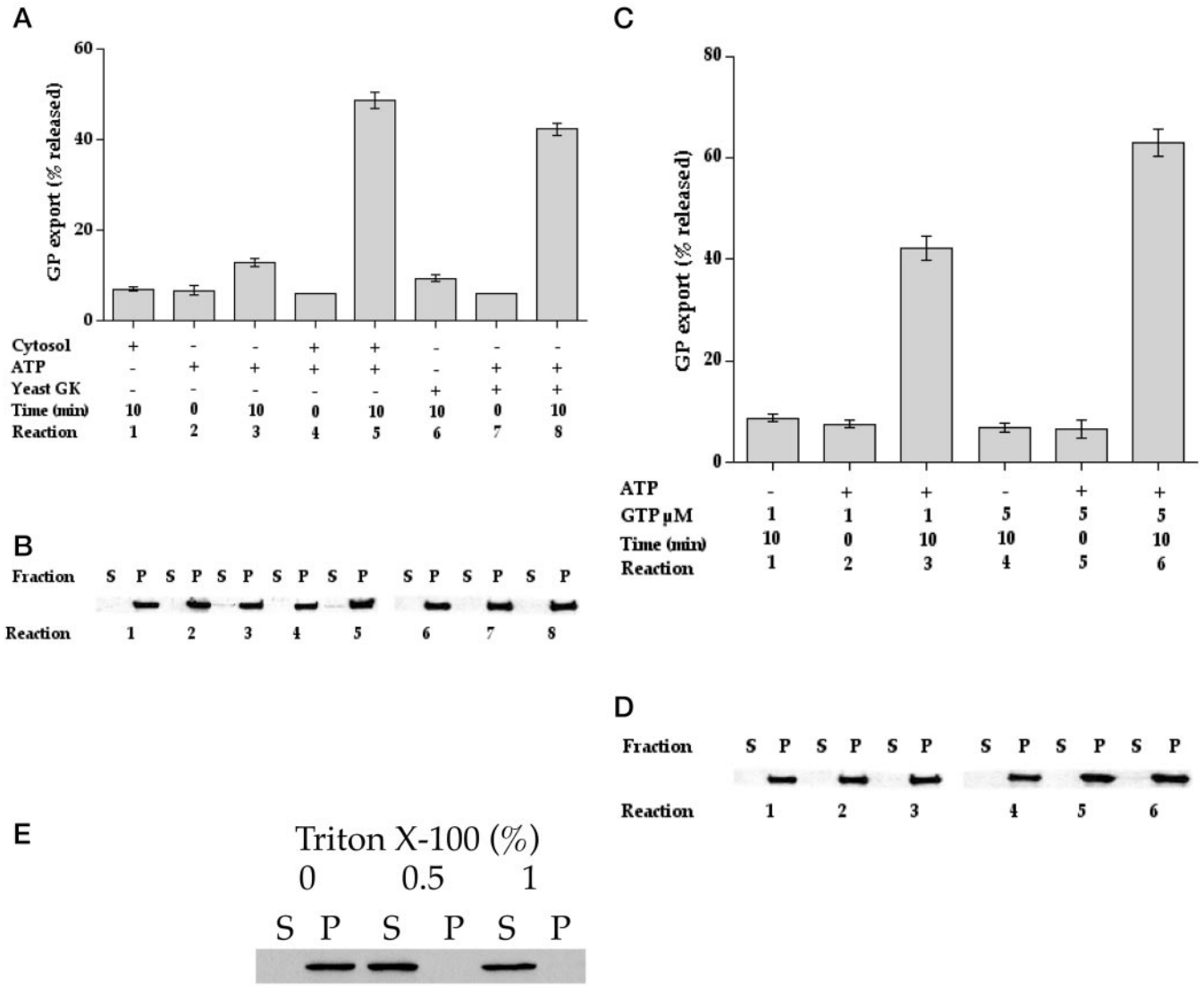

FIG. 6. **Membrane integrity is maintained during glycopeptide export.** Quantitation of both glycopeptide and PDI present in the membrane and supernatant fractions was performed for each reaction as described under "Experimental Procedures." *A*, glycopeptide (*GP*) export using partially purified rat liver cytosolic factor and yeast GK. *Reactions 5* and *8* contained the ATP-regenerating system and either rat cytosol or GK, respectively, and were incubated for 10 min at 32 °C. Other reactions (*reactions 1–4*, *6*, and *7*) are controls that show only background release and confirm that glycopeptide export requires ATP, cytosol, or GK and incubation at physiological temperature (compare the controls *versus Reactions 5* and *8*). Reactions were performed in duplicate, and data are presented as means ± S.E. *B*, compartmentalization of the ER luminal protein PDI in the reactions in *A*. No significant release of PDI into the supernatant (designated *S* and *P* for soluble and pellet, respectively) was observed in any of the reactions, including those (*reactions 5* and *8*) where glycopeptide export was ~50%, indicating that the integrity of the membranes was not compromised under the glycopeptide export conditions. *C*, glycopeptide export in the presence of the ATP-regenerating system and 1 or 5 μM GTP with no cytosolic proteins added. GTP at 1 or 5 μM and in the presence of an ATP-regenerating system and incubation at 32 °C for 10 min resulted in 45 and 70% glycopeptide export (*reactions 3* and *6*, respectively). GTP on its own (*reactions 1* and *4*) or GTP and ATP incubated on ice for 10 min (*reactions 2* and *5*) show background release of glycopeptide. Reactions were performed in duplicate, and data are presented as means ± S.E. *D*, compartmentalization of the ER luminal protein PDI in the reactions in *C*. No release of PDI was observed under conditions that allowed 45 and >70% export (*reactions 3* and *6*, respectively). The experiment was performed done twice, with essentially identical results. *E*, confirmation that PDI is released into the medium once membrane integrity has been compromised by treating the microsomes with Triton X-100. In the control sample (0%), PDI was exclusively localized to the membrane fraction (*P*); however, when Triton X-100 was added (0.5 and 1%), the protein was quantitatively released into the medium (*S*).

the GTPase activity is microsomal and hence may potentially interact directly with the glycopeptide export channel. It is worth noting here that a large number of studies have been performed using this ATP-regenerating system (*i.e.* containing GDP-Man) in *in vitro* systems to study transport across membranes. Hence, the probability of generating GTP upon addition to membranes must be considered under these conditions.

The kinetics of glycopeptide export from rat liver microsomes in the presence of ATP and GTP are rapid, with a half-life for glycopeptide exported of ~3 min. The export half-life of the MHC class I heavy chain from the ER to the cytosol in cells expressing the human cytomegalovirus genes U2 and US11 is also in the range of 2–3 min and is therefore consistent with those of other substrates (13). Additionally, efflux of oligopeptides from microsomes during antigen presentation is independent of TAP, requires ATP, and follows relatively rapid kinetics (16, 17). On the other hand, misfolded protein degradation and oligosaccharide export are slower than for glycopeptides (7, 11, 12). This is presumably because misfolded proteins enter folding and quality control pathways distinct from simple molecules like glycopeptides, and they may also require unfolding before being transported back to the cytosol.

We have also demonstrated that a trypsin-sensitive component on the microsomal membrane is essential for export of

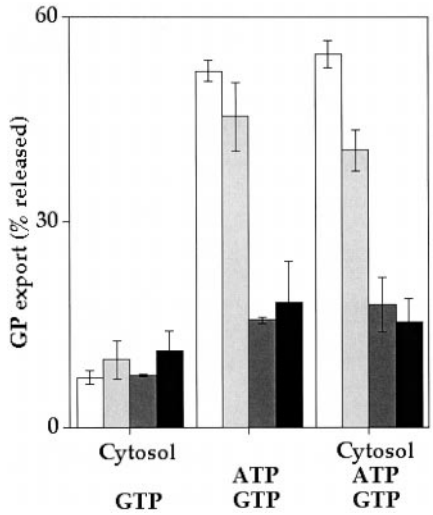

FIG. 7. **Glycopeptide export from rat liver microsomes is sensitive to trypsin treatment.** Microsomes were stripped with salt and incubated without (*white bars*) or with 1, 2.5, or 10 μg/ml trypsin (*light-gray* to *black bars*) for 10 min on ice. The membranes were then washed twice with buffer and assayed for glycopeptide export competence in the presence of 10 μM GTP and cytosol, ATP, or both as indicated. The experiment was performed in duplicate.

glycopeptides. This second export factor could be the same as the GTPase; but alternatively, these two factors could be distinct. The present data do not allow us to distinguish between the two possibilities. The latter interpretation gives rise to the interesting possibility that the GTPase is a regulatory factor, whereas the protease-sensitive molecule may be a channel or other structural component. Sensitivity of export to trypsin digestion indicates that at least part of the system is located on the cytosolic face of the ER membrane, and this may be the cytoplasmically orientated loops of a channel protein. Very recent data from studies on *S. cerevisiae* indicate that glycopeptide export is mediated by Sec61p (38). Hence, a possible role for GTP is in regulating opening of the Sec61p translocon, but the identity of this GTPase is currently unknown. Our data suggest that it is unlikely to be signal recognition particle receptor α, although we cannot rule out a very small, but critical proteolytic inactivation that may not have been detected by our analysis. In addition, the role of the transmembrane β subunit has not been addressed; but as the mammalian ER membrane contains numerous GTPases, there are many other candidates for this role. It is worth noting that GTP did not substitute for cytosol in glycopeptide export from *S. cerevisiae* semi-intact cells (data not shown), indicating that at least this aspect of the pathway is likely restricted to metazoans. The identification of both the microsomal GTPase identified here and the glycopeptide translocon, presumed to be Sec61p in mammalian systems, is clearly a vital requirement for complete characterization of glycopeptide export, for addressing the relationship of this process to the better characterized Sec61p-mediated protein retranslocation pathway, and finally for understanding the role this mechanism plays in antigen presentation by MHC class I.

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