The EMBO Journal vol.15 no.24 pp.6931–6942, 1996

A microsomal ATP-binding protein involved in efficient protein transport into the mammalian endoplasmic reticulum

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Protein transport into the mammalian endoplasmic reticulum depends on nucleoside triphosphates. Photoaffinity labelling of microsomes with azido-ATP prevents protein transport at the level of association of precursor proteins with the components of the transport machinery, Sec61α and TRAM proteins. The same phenotype of inactivation was observed after depleting a microsomal detergent extract of ATP-binding proteins by passage through ATP–agarose and subsequent reconstitution of the pass-through into proteoliposomes. Transport was restored by co-reconstitution of the ATP eluate. This eluate showed eight distinct bands in SDS gels. We identified five lumenal proteins (Grp170, Grp94, BiP/Grp78, calreticulin and protein disulfide isomerase), one membrane protein (ribophorin I) and two ribosomal proteins (L4 and L5). In addition to BiP (Grp78), Grp170 was most efficiently retained on ATP–agarose. Purified BiP did not stimulate transport activity. Sequence analysis revealed a striking similarity of Grp170 and the yeast microsomal protein Lhs1p which was recently shown to be involved in protein transport into yeast microsomes. We suggest that Grp170 mediates efficient insertion of polypeptides into the microsomal membrane at the expense of nucleoside triphosphates.

Keywords: ATP/Grp170/Lhs1p/mammalian microsomes/ protein transport

Introduction

Export of the vast majority of newly synthesized proteins from mammalian cells requires a signal sequence at the amino-terminus of these presecretory proteins. This signal peptide-dependent transport (for review see Meyer, 1991; Rapoport, 1992; Klappa *et al.*, 1993) is initiated at the level of the membrane of the endoplasmic reticulum (ER). It includes various steps such as association of the precursor protein with the ER membrane (also termed targeting reaction), membrane insertion and completion of transport (translocation reaction). The signal sequences not only facilitate membrane specificity, but also help to preserve the transport-competent (non-native) state of the precursor proteins.

Transport competence in the cytosol is maintained by two alternative mechanisms (Zimmermann and Meyer, 1986; Wiech *et al.*, 1991; Klappa *et al.*, 1993). In the first mechanism, involving two ribonucleoparticles (RNPs), protein synthesis is slowed down. In the second mechanism, involving molecular chaperones but no RNPs, protein folding and/or aggregation is slowed down. The RNP-dependent or co-translational pathway involves the ribosome (Perara *et al.*, 1986; Hedge and Lingappa, 1996) and the signal recognition particle (SRP) (Walter and Blobel, 1981a,b; Walter *et al.*, 1981; Bernstein *et al.*, 1989; Römisch *et al.*, 1989), the latter stalling translation activity of the first upon binding to the emerging signal peptide. The two RNPs make contact to the microsomal surface via an SRP receptor (docking protein) (Meyer and Dobberstein, 1980a,b; Gilmore *et al.*, 1982a,b; Meyer *et al.*, 1982; Lauffer *et al.*, 1985; Tajima *et al.*, 1986) and a putative ribosome receptor (Savitz and Meyer, 1990, 1993; Wanker *et al.*, 1995). Transport of RNP-dependent precursor proteins, such as preprolactin, requires GTP due to two GTP-binding proteins, the SRP 54 kDa subunit and docking protein α-subunit (Connolly and Gilmore, 1986, 1989; Connolly *et al.*, 1991; Rapiejko and Gilmore, 1992; Bacher *et al.*, 1996). The RNP-independent or post-translational pathway involves Hsc70 and at least one additional cytosolic protein which may be identical to Hsp40 (Wiech *et al.*, 1987, 1993; Zimmermann *et al.*, 1988). These molecular chaperones bind to fully synthesized precursor proteins keeping them in a loosely folded conformation that also is promoted by the presence of the signal sequence. The transport of RNP-independent precursor proteins, such as preprocecropin A, requires no GTP, but ATP that is hydrolysed by Hsc70.

We assume that the RNP-dependent and independent pathways converge at the level of a putative signal peptide receptor which could be a subunit of a general translocase in the ER membrane (Klappa *et al.*, 1994; Jungnickel and Rapoport, 1995). The essential constituents of this translocase on the molecular level are the TRAM protein (Translocating chain Associating Membrane protein) (mp39) (Wiedmann *et al.*, 1987b; Krieg *et al.*, 1989; Thrift *et al.*, 1991; Görlich *et al.*, 1992a) and the Sec61p complex, comprising Sec61αp (p37, imp34), Sec61βp and Sec61γp (High *et al.*, 1991, 1993; Kellaris *et al.*, 1991; Görlich *et al.*, 1992b; Görlich and Rapoport, 1993; Hartmann *et al.*, 1994).

Evidence has accumulated that an additional nucleoside triphosphate-utilizing subunit takes part in both transport mechanisms (Klappa *et al.*, 1991; Zimmerman and Walter, 1991; Zimmermann *et al.*, 1991). We have shown pre-

Table I. Protein transport activities of various microsomes and proteoliposomes

	ppl transport	ppl-86mer X36 kDa	ppcecA transport	pc transport
RM	yes	yes	yes	yes
$RM_{trypsin}$	no	no	yes	yes
$RM_{trypsin}$ + DP_f	yes	yes	n.d.[c]	n.d.
$RM_{azido\text{-}ATP}$	reduced	reduced	reduced	yes
$RM_{trypsin+azido\text{-}ATP}$ + DP_f	reduced	reduced	n.d.	n.d.
Control liposomes	yes	yes	n.d.	yes
–ATP-eluate liposomes[a]	reduced	reduced	n.d.	yes
+ATP-eluate liposomes[b]	yes	yes	n.d.	yes

The capabilities of dog pancreas microsomes (RM) to process and sequester (i.e. transport) preprolactin (ppl), preprocecropinA (ppcecA) and M13 procoat protein (pc) in cell-free translation systems, derived from rabbit reticulocytes, was described previously (Wiech *et al.*, 1987; Schlenstedt *et al.*, 1990). Furthermore, it was shown previously that under these conditions (Zimmermann *et al.*, 1991) a nascent preprolactin chain (ppl-86mer) becomes associated with and can be crosslinked to microsomal membrane proteins with an approximate mass of 36 kDa (ppl-86mer X36 kDa), most likely representing the glycoprotein TRAM protein (mp39) (Wiedmann *et al.*, 1987b; Krieg *et al.*, 1989; Görlich *et al.*, 1992a) and the unglycosylated Sec61α protein (p37, imp34) (High *et al.*, 1991; Kellaris *et al.*, 1991; Görlich *et al.*, 1992b). The effects of pretrypsinization of microsomes and of reconstitution of these microsomes with elastase fragment of the α-subunit of the docking protein (DP_f) (Meyer and Dobberstein, 1980a) was described elsewhere (Schlenstedt *et al.*, 1990; Zimmermann *et al.*, 1991; Klappa *et al.*, 1993). The effect of photoaffinity modification of microsomes with azido-ATP was described previously (Klappa *et al.*, 1991; Zimmermann *et al.*, 1991). The transport activities of proteoliposomes made from a total detergent extract of microsomes (control) or from the pass-through of ATP–agarose co-reconstituted or not with the ATP-eluate, as shown in Figures 2, 4 and 5, are compared with those activities of RM and of pretrypsinized RM that were modified by azido-ATP and reconstituted with the DP_f.

[a]Liposomes prepared from pass-through –ATP-eluate.

[b]Liposomes prepared from pass-through +ATP-eluate.

[c]n.d., not determined.

viously that RNP-independent transport of purified and denatured preprocecropin A depends on the hydrolysis of ATP, even in the absence of cytosolic chaperones, and on a microsomal protein which is sensitive to photoaffinity labelling with azido-ATP (C^8-azido-adenosine-5′-triphosphate) (Table I). In addition, RNP-dependent transport of preprolactin also involves a microsomal protein which is sensitive to photoaffinity labelling with azido-ATP. In this case we analysed at what stage RNP-dependent transport is affected (Zimmermann *et al.*, 1991) by investigating transport intermediates of a nascent preprolactin chain (ppl-86mer) as described by Connolly and Gilmore (1986). After photoaffinity labelling of microsomes with azido-ATP productive binding of ppl-86mer to microsomes was no longer possible. Azido-ATP prevented: (i) protease-resistant binding of ppl-86mer; (ii) crosslinking to what represents a mixture of TRAM- and Sec61α-protein (Görlich *et al.*, 1992b); and (iii) chase to sequestered pl-56mer following release from the ribosome with puromycin. The same results were obtained with pretrypsinized microsomes that, after photolabelling, were reconstituted with the cytosolic domain of the α-subunit of docking protein, thereby excluding docking protein (α-subunit) as the target of azido-ATP. Thus, the ATP-requiring component in RNP-dependent transport acts past docking protein and before the TRAM–Sec61 complex. Photolabelling with azido-ATP, if carried out after binding of the ppl-86mer, did not give rise to protease sensitivity of ppl-86mer and did not inhibit chase to sequestered pl-56mer.

We asked whether the solubilization of the transport machinery with detergent and subsequent functional reconstitution into liposomes (Nicchitta and Blobel, 1990) can be developed into an assay for the purification of the azido-ATP-sensitive transport component. For this purpose, we tried to deplete a microsomal detergent extract of ATP-binding proteins by chromatography on ATP–agarose. Here, we show the feasibility of this approach and describe the ATP-binding proteins. Functional analysis led to the conclusion that the azido-ATP-sensitive component is among those ATP-binding proteins.

Results

Protein transport into the mammalian endoplasmic reticulum depends on nucleoside triphosphates and can be inactivated by photoaffinity modification of microsomes with azido-ATP (see Introduction). To identify ATP-binding protein(s) responsible for these findings it was a prerequisite to develop a suitable assay, comprising proteoliposomes that are reconstituted from solubilized microsomal membranes.

Functional reconstitution of microsomal protein transport

The reconstitution method of Nicchitta and Blobel (1990) was modified with respect to the solubilization of salt-washed microsomes (KRM). For this purpose we used the zwitterionic detergent CHAPS in the presence of glycerol and egg yolk phospholipids (see Materials and methods). Following dialysis of the detergent extract and sedimentation of proteoliposomes, the reconstituted protein transport activity was measured as processing of *in vitro*-synthesized precursor proteins and sequestration of the corresponding mature proteins.

We employed preprolactin and M13 procoat protein as transport substrates. Since in microsomes inhibition by azido-ATP was observed for the transport of preprolactin, but not for the membrane assembly of procoat protein (cf. Table I), the latter served as a control for non-specific effects. For both substrates, the processing efficiency of proteoliposomes within 60 min, measured as production of mature prolactin or coat protein, respectively approached that of native microsomal membranes (Figure 1), disregarding, however, that 1 μl of proteoliposomes had been derived from 2.5 μl of KRM. On the other hand,

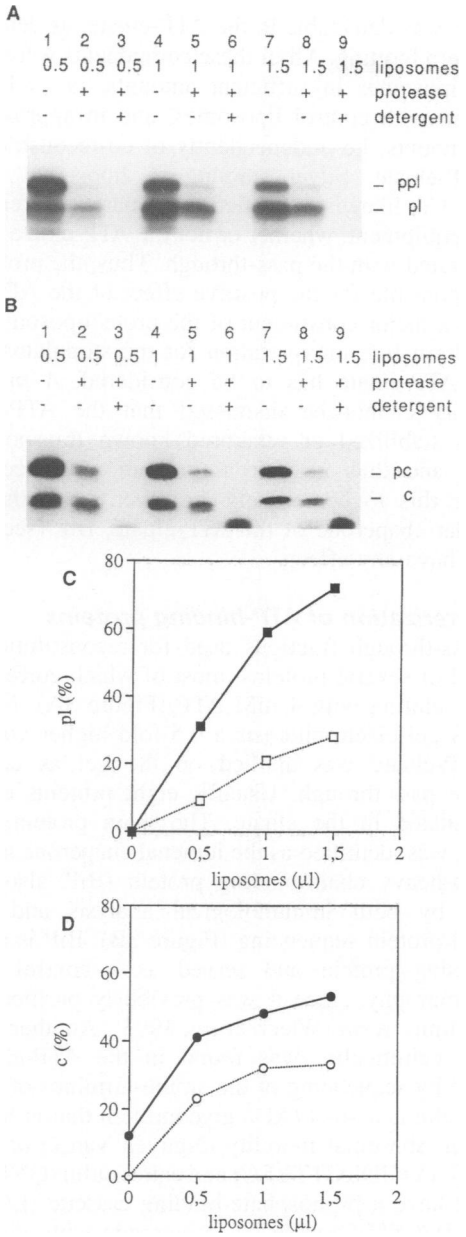

Fig. 1. Functional reconstitution of microsomal protein transport. Proteoliposomes (15 µl) were prepared from a total detergent extract of KRM (37.5 µl), as described in Materials and methods, and tested for protein transport, i.e. processing and sequestration, activity. For this purpose, the presecretory protein preprolactin (ppl) and the precursor protein M13 procoat protein (pc) were synthesized for 60 min at 30°C in rabbit reticulocyte lysates in the presence of [^{35}S]methionine and microsomes (data not shown) or proteoliposomes (given in µl/6.25 µl of translation reaction). Then the samples were divided and incubated in the absence (–) or presence (+) of proteinase K, or in the presence of proteinase K plus detergent for 60 min at 0°C. All samples were analysed by gel electrophoresis, fluorography (**A** and **B**) and densitometry (**C** and **D**). The amounts of the processed forms (■, pl and ●, c) and sequestered processed forms (□, ○) are given as percent of precursor plus processed form. Note that in the case of pc (three methionines) and c (one methionine) the distribution of methionines was taken into account for the calculation of processing and sequestration efficiencies. In the case of microsomes the following values were determined: pl, 86%; sequestered pl, 73% (for 1 µl/6.25 µl); c, 43%; sequestered c, 33% (for 0.5 µl/6.25 µl).

the sequestration efficiency, measured as protection of the mature protein against proteinase K, was clearly lower than in microsomes. Approximately one-third of procoat protein and one- to two-thirds of preprolactin were processed without being sequestered (Figures 1, 2 and 4). In the case of procoat protein this effect was, at least partially, explained by an endogenous processing activity of the reticulocyte lysate (Figure 1D, 0 µl liposomes), as was described earlier (Wiech *et al.*, 1987). Therefore only sequestration of coat protein was taken as a reliable indicator of transport efficiency. In the case of preprolactin, processing without sequestration obviously was due to a subpopulation of signal peptidase molecules reconstituted with inside-out orientation. Alternatively, a fraction of preprolactin polypeptides was not completely translocated and upon signal peptide cleavage could transit from the translocation apparatus back to the *cis*-side of the membrane (Nicchitta and Blobel, 1993). Nevertheless, a linear dependence of preprolactin processing and prolactin sequestration on the amount of liposomal membranes was observed in a range up to 1.5 µl liposomes per translation reaction.

ATP-binding proteins are involved in reconstituted protein transport

In order to deplete KRM of ATP-binding proteins a microsomal detergent extract was applied to ATP-C^{8}–agarose equilibrated with a buffer that was similar in composition to the solubilization buffer (see Materials and methods). By using $MnCl_2$ in place of $MgCl_2$, i.e. by transforming ATP–agarose to the Mn-form, the binding of some proteins to the column was improved. Following a high-salt (400 mM KCl) and a low-salt (0 mM KCl/4–10 mM $MnCl_2$ or $MgCl_2$) wash, bound proteins were eluted with steps of 4 mM ATP (400 mM KCl) and 10 mM ATP (2 M KCl). The fraction eluting with 4 mM ATP is referred to as ATP-eluate. Usually not more than 4% of the protein applied to the column was found in this ATP-eluate.

Figure 2 demonstrates that proteoliposomes, when made from the pass-through fractions (see Materials and methods), showed a clear reduction of transport of preprolactin as compared with control liposomes made from a total detergent extract. Co-reconstitution of the pass-through with the ATP-eluate, on the other hand, led to reactivation; prolactin sequestration was stimulated by a factor of 4 (Figure 2A). Figure 2C shows the results obtained after a similar chromatography in more detail. It became obvious from these experiments that the stimulation of preprolactin transport by the ATP-eluate depended on the amount of ATP-eluate co-reconstituted with the pass-through. No inhibitory (minus ATP-eluate) or stimulatory (plus ATP-eluate) effect was observed for the transport of procoat protein (Figure 2B and D).

It has to be noted that: (i) all proteoliposomes were made from equal amounts of protein (cf. Figure 3A), usually 50–80 µg for 15 µl proteoliposomes, the eluate contributing only 3–5 µg; (ii) reconstitution was performed under identical conditions, i.e. under adjustment of protein dilution and buffer composition; and (iii) reconstitution of the ATP-eluate alone did not result in transport-competent proteoliposomes (not shown).

None of the microsomal components known to be directly involved in protein transport, namely docking protein (α- and β-subunit), Sec61α protein, TRAM protein, signal peptidase (SPC23) or the putative ribosome receptor

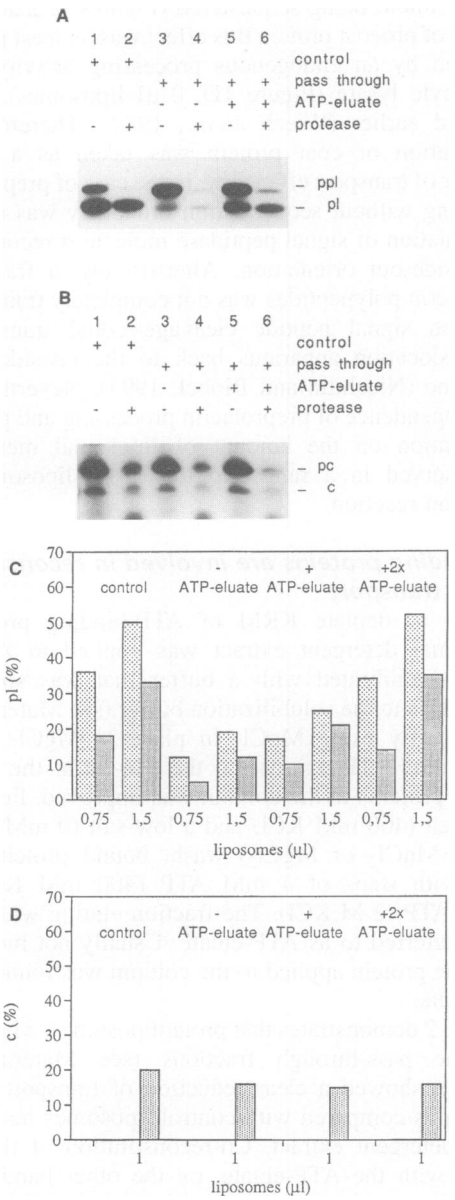

Fig. 2. Microsomal ATP-binding proteins are involved in the transport of preprolactin into proteoliposomes. The precursor proteins preprolactin (ppl) and procoat protein (pc) were synthesized for 60 min at 30°C in rabbit reticulocyte lysates in the presence of [^{35}S]methionine and proteoliposomes, which were derived from a total microsomal extract (control), an ATP–agarose pass-through (–ATP-eluate), a pass-through co-reconstituted with ATP-eluate (+ATP-eluate) or a pass-through co-reconstituted with twice the amount of ATP-eluate [+2× ATP-eluate in (C) and (D)]. The samples were then divided and incubated in the absence (–) or presence (+) of proteinase K for 60 min at 0°C. All samples were analysed by gel electrophoresis, fluorography and densitometry. Note that the proteoliposomes were reconstituted from protein fractions obtained by chromatography either on Mn-ATP–agarose (A and B) or on Mg-ATP–agarose (C and D). Different volumes of proteoliposomes per 6.25 µl of translation reaction were used: 0.75 µl (**A**), 0.38 µl (**B**), 0.75 and 1.5 µl (**C**), 1 µl (**D**). In (A), the amounts of sequestered pl were as follows: 46% (lane 2), 6% (lane 4), 28% (lane 6); in (B), amounts of sequestered c were: 14% (lane 2), 11% (lane 4), 11% (lane 6). In (C) and (D), the amounts of the processed forms (pl, left bar) and sequestered processed forms (pl and c, right bar) are given as percent of precursor plus processed form, considering the distribution of methionines (see Figure 1).

ERp180, was detectable in the ATP-eluate, as determined by Western blotting. All of these components were present in the liposomes in sufficient amounts, i.e. >100% as compared with control liposomes, and in approximately equal amounts, i.e. independently of co-reconstitution of the ATP-eluate. Silver staining of liposomal proteins (Figure 3A) likewise revealed no apparent difference in protein equipment, whether or not the ATP-eluate was co-reconstituted with the pass-through. Thus, the protein that was responsible for the positive effect of the ATP-eluate was not a major constituent of the proteoliposomes.

An alternative interpretation for the stimulatory effect of the ATP-eluate has to be considered. *A priori* the possibility cannot be dismissed that the ATP-binding proteins stabilized or renatured known transport components and thus affected translocation indirectly. We consider this to be unlikely, however, since the major molecular chaperone of the ATP-eluate, BiP (see below) did not have any effect.

Characterization of ATP-binding proteins

The pass-through fractions used for reconstitution were depleted of several proteins, most of which corresponded to those eluting with 4 mM ATP (Figure 3A). Note that for SDS gel electrophoresis a 6.5-fold higher volume of the ATP-eluate was applied on the gel as compared with the pass-through. Usually, eight proteins could be discriminated in the eluate. The main protein, termed ABP78, was identified as the lumenal chaperone immunoglobulin-heavy chain-binding protein (BiP, also termed Grp78) by both immunological analysis and amino-terminal protein sequencing (Figure 3B). BiP is an ATP-hydrolysing protein and served as a control for the chromatography, since it was previously purified on the same affinity resin (Wiech *et al.*, 1993). Another lumenal protein, calreticulin, was found in the ATP-eluate, as revealed by sequencing of the amino-terminus of ABP57. Calreticulin is a 46–49 kDa glycoprotein that in SDS gels shows an abnormal mobility (Nguyen Van *et al.*, 1989). Both BiP (VGIDLGTTYSC) and calreticulin (QNIDCGGGYVK) have a β-phosphate-binding cassette (L/I/VxL/I/VDxGS/T/GS/T/GxxK/R/C) in common with other ATP-binding proteins (Flaherty *et al.*, 1991). This cassette also was found in the sequenced amino-terminus of ABP150 (MSVDLGSESMK). This protein turned out to be identical with Grp170 (Chen *et al.*, 1996). A fourth lumenal chaperone, Grp94, was identified by sequencing BrCN peptides of ABP103. Grp94, like its cytosolic counterpart Hsp90 (Csermely and Kahn, 1991), has triphosphate- and adenine-binding sequences. Hsp90 was described to bind to ATP–agarose in the presence of Ca^{2+} or Mn^{2+} ions (Csermely and Kahn, 1991). Finally, also the lumenal protein disulfide isomerase (PDI) was detected in the ATP-eluate (ABP52, Figure 3B). The appearance of PDI, which lacks phosphate- and nucleoside-binding sequences, in the ATP-eluate may have been due to its ability to interact with calreticulin (Baksh *et al.*, 1995).

Considering the results of carbonate extraction and Triton X-114 phase separation experiments (data not shown), we concluded that only one protein present in the ATP-eluate is a membrane protein, namely ABP67. Subsequently ABP67 was identified as ribophorin I, both by amino-terminal sequencing and by Western analysis.

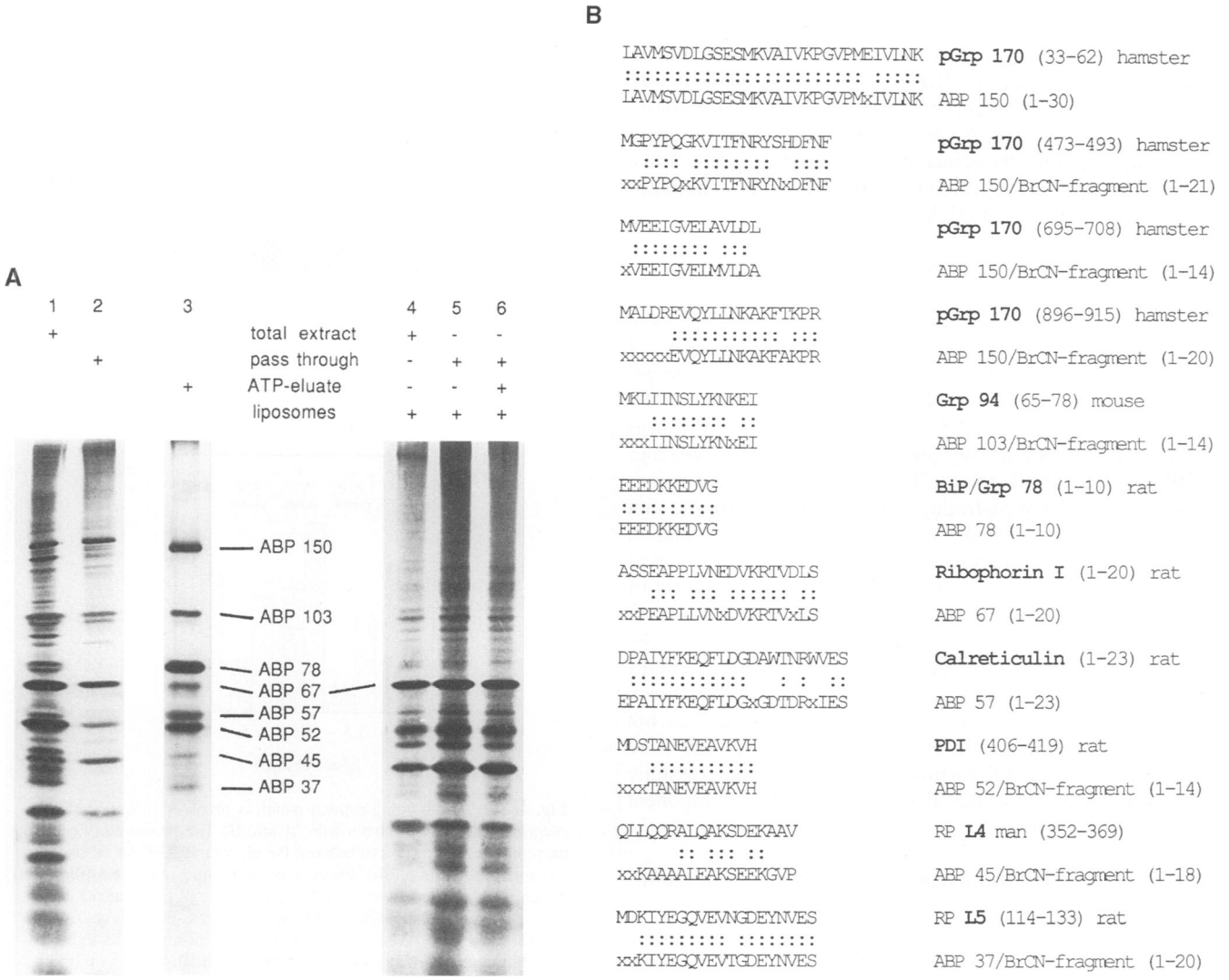

Fig. 3. Microsomal ATP-binding proteins (ABPs) retained on ATP–agarose. (**A**) The protein composition of a total detergent extract of KRM (1 μl), the ATP–agarose pass-through (5 μl), the ATP-eluate (32 μl), and of proteoliposomes (1 μl) which were derived from the indicated fractions was analysed by SDS–polyacrylamide gel electrophoresis and silver staining. The proteoliposomes were tested for protein transport activity as in Figure 2A and B. The position and the apparent molecular mass of the ABPs, present in the ATP-eluate, are indicated. The position of ABP67, which is identical to ribophorin I, was determined by immunological techniques and is indicated also for proteoliposomes. Lanes 1 and 2, lane 3 and lanes 4–6, respectively, were run on three separate gels, thus explaining minor differences in the electrophoretic separation. (**B**) The ABPs were analysed either directly or after cyanogen bromide cleavage by protein sequencing. The sequences were subjected to database (SWISSPROT) searching (FASTA, Pearson and Lipman, 1988) and were checked for the presence of a consensus nucleotide β-phosphate-binding sequence as found in Hsc70, actin and hexokinase (Flaherty *et al.*, 1991). We positively identified ABP150 as Grp170, ABP78 (as BiP/Grp78), ABP103 (as Grp94/Erp99), ABP67 (as ribophorin I), ABP57 (as calreticulin), ABP52 (as PDI), ABP45 as ribosomal protein (RP) L4, and ABP37 as ribosomal protein (RP) L5 on the basis of molecular mass and primary structure identity (Edman *et al.*, 1985; Munro and Pelham, 1986; Chan *et al.*, 1987; Harnik-Ort *et al.*, 1987; Mazzarella and Green, 1987; Murthy *et al.*, 1990; Bagni *et al.*, 1993; Chen *et al.*, 1996). We note that the identity of ABP45 with ribosomal protein L4 became apparent only after sequencing of the corresponding cDNA (see Materials and methods). The cβ-PBS alignment allowed us to identify ABP150, ABP78, ABP67 and ABP57 as potential nucleotide-binding proteins. Furthermore, Grp94/Erp99 was shown to contain putative triphosphate and adenine-binding motifs (Mazzarella and Green, 1987; Csermely and Kahn, 1991). Note that: (i) two dots represent an identical amino acid residue present in the respective ABPand the corresponding protein in the data base; (ii) x represents an ambiguous result in the amino acid analysis in the case of ABPs; (iii) the first x in the case of cyanogen bromide (BrCN) fragments was added to the amino-terminus of the fragment assuming that a fragment starting with methionine was subjected to sequencing.

Also ribophorin I contains a sequence resembling a truncated β-phosphate-binding site (VALDPGSKISI) which would reside in the lumenal domain (Crimaudo *et al.*, 1987) and could be involved in binding of the dolichol pyrophosphate-linked oligosaccharide.

BrCN fragmentation of two proteins, which eluted with 10 mM ATP/2 M KCl and which showed electrophoretic mobilities identical to those of ABP45 and ABP37, respectively, led to partial amino acid sequences. ABP37 was identified as ribosomal protein L5 while ABP45 showed no significant homologies to sequences stored in the SWISSPROT database. After sequencing of the corresponding cDNA, however, ABP45 was identified as ribosomal protein L4.

In order to reduce the number of ATP-binding proteins that could be responsible for transport stimulation, we tried to quantitate the degree of depletion of these proteins after passage through ATP–agarose. With the help of antibodies, and by estimating the protein amounts on silver-stained gels, we found that only small proportions

of total ribophorin I (12%), Grp94 (5%), calreticulin (12%) and PDI (12%) bound to ATP–agarose. However, nearly quantitative binding for BiP (96%) and efficient binding of ABP150 were observed (50–70%). The early pass-through fractions which were used for reconstitution were even more efficiently depleted of these two proteins. Thus, these two proteins appeared to us as the primary candidates for the positive effect of the ATP-eluate.

BiP is not sufficient to substitute for ATP-binding proteins

From the proteins present in the ATP-eluate only the lumenal BiP was previously described to play a direct role in protein translocation into the ER of yeast (Vogel *et al.*, 1990; Nguyen *et al.*, 1991; Sanders *et al.*, 1992; Brodsky *et al.*, 1993, 1995). Particularly since BiP was the main protein in our preparation, we asked whether BiP can substitute for the ATP-eluate. The proteoliposomes made from the pass-through were significantly, but not completely inactivated, as determined by preprolactin transport assays (Figure 4A and B). Reactivation was observed upon co-reconstitution of ATP-eluate, but not of purified BiP, even when not supplementing more BiP than present in the ATP-eluate or in the total extract. The functional state of purified BiP was tested by measurements of ATP hydrolysis and peptide-binding capacity (Wiech *et al.*, 1993). These data show that BiP *per se* was not able to substitute for the ATP-binding proteins that were depleted from the microsomal extract by chromatography on ATP–agarose, i.e. that BiP is not the active component in the ATP-eluate.

ATP-binding proteins are involved early in protein translocation

It has previously been shown (Zimmermann *et al.*, 1991) that after photoinactivation of microsomes with azido-ATP, nascent preprolactin did not become associated with the Sec61–TRAM protein complex (Görlich *et al.*, 1992a,b). This was elucidated by reduced chemical crosslinking of a preprolactin fragment (ppl-86mer), arrested at the ribosome due to truncation of the corresponding mRNA, to the Sec61–TRAM complex (Table I). When we carried out similar crosslinking experiments with proteoliposomes depleted of ATP-binding proteins, we also found that the association of ppl-86mer with this complex (ppl-86mer X 36 kDa) was reduced by a factor of three as compared with control liposomes (Figure 5, compare lanes 1 and 4). On the other hand, the association with the 54 kDa subunit of signal recognition particle (SRP54) was unchanged or, as found in other experiments (not shown), even increased, as might be explained by different efficiencies of co-sedimentation of liposomes and ppl-86mer bound to SRP54 (see Materials and methods). Correspondingly, the production of the mature pl-56mer fragment by these proteoliposomes was ~3-fold less efficient as compared with control liposomes. This became evident after releasing the ppl-86mer from the ribosome by addition of puromycin (Figure 5, compare lanes 2 and 3 with lanes 5 and 6). Both inhibition of association with Sec61αp–TRAMp and of chase to pl-56mer no longer were observed when using proteoliposomes co-reconstituted from the pass-through plus the ATP-eluate (lanes 7–9). These findings strongly suggest that ATP-binding proteins are acting at an early step of protein translocation, i.e. between signal recognition particle/docking protein and Sec61p complex/TRAM protein.

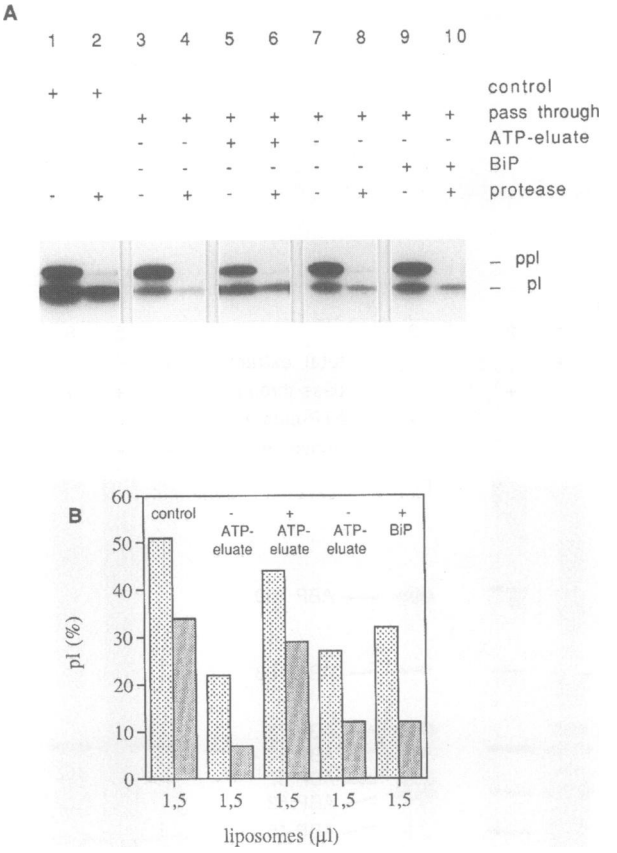

Fig. 4. The ATP-binding protein which is involved in transport of preprolactin is distinct from BiP. (**A** and **B**) The presecretory protein preprolactin (ppl) was synthesized for 60 min at 30°C in rabbit reticulocyte lysates in the presence of proteoliposomes (1.5 µl/6.25 µl) which were derived from a total microsomal extract (control), an ATP–agarose pass-through (–ATP-eluate), a pass-through co-reconstituted with ATP-eluate (+ATP-eluate) or a pass-through co-reconstituted with 15 µg of purified BiP (+ BiP). BiP was purified according to Wiech *et al.* (1993). The samples were then divided and incubated in the absence (–) or presence (+) of proteinase K for 60 min at 0°C. All samples were analysed by gel electrophoresis, fluorography (A) and densitometry (B). Note that the proteoliposomes tested in lanes 1–6 and 7–10, respectively, were prepared on two consecutive days. For this reason, the pass-through was reconstituted twice. The control liposomes on each day showed nearly identical protein transport activity. In (B), the amount of the processed form (pl, left bar) and sequestered processed form (right bar) are given as percent of precursor plus processed form.

Discussion

There are consecutive steps of nucleoside triphosphate hydrolysis that accompany the transport of presecretory proteins into the ER of yeast, as well as of mammalian cells. Considering both the RNP-dependent and -independent mechanism, the following ATP- or GTP-binding proteins have been described to participate in the overall transport process: (i) at the cytosolic side, Hsc70 (Chirico *et al.*, 1988; Deshaies *et al.*, 1988; Zimmermann *et al.*, 1988; Wiech *et al.*, 1993) and SRP (54 kDa subunit) (Connolly and Gilmore, 1986, 1989; Hann *et al.*, 1989; Hann and Walter, 1991); (ii) at the level of the ER

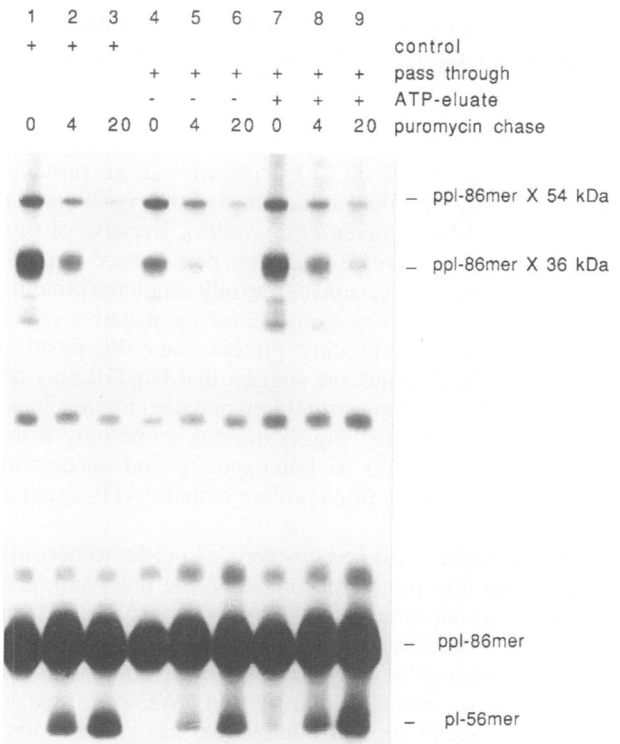

Fig. 5. The ATP-binding protein involved in transport of preprolactin acts before TRAM and sec61α protein. The nascent presecretory protein ppl-86mer was synthesized for 20 min at 30°C in rabbit reticulocyte lysates in the presence of proteoliposomes (0.67 μl/6.25 μl) which were derived from a total microsomal extract (control), an ATP–agarose pass-through or a pass-through co-reconstituted with ATP-eluate. After supplementation with puromycin and further incubation for 0, 4 and 20 min (chase) aliquots were subjected to crosslinking with succinimidyl-4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate. All samples were analysed by gel electrophoresis, fluorography and densitometry. The positions (i) of crosslinks between ppl-86mer and the SRP 54 kDa-subunit (ppl-86mer X 54 kDa) (Krieg *et al.*, 1986; Kurzchalia *et al.*, 1986; Wiedmann *et al.*, 1987a); (ii) of crosslinks between ppl-86mer and TRAM protein and Sec61α-protein, respectively (ppl-86mer X 36 kDa) (Wiedmann *et al.*, 1987b; Krieg *et al.*, 1989; Görlich *et al.*, 1992a,b), as well as (iii) of ppl-86mer and pl-56mer are indicated. For transport of full-length preprolactin into these proteoliposomes see Figure 4.

membrane, docking protein (α-subunit) (Ogg and Walter, 1992; Rapiejko and Gilmore, 1992) and, albeit not directly involved, the ATP carrier (Mayinger and Meyer, 1993; Mayinger *et al.*, 1995); (iii) at the lumenal side, BiP (Vogel *et al.*, 1990; Brodsky *et al.*, 1993, 1995; Lyman and Schekman, 1995; Panzner *et al.*, 1995) and Lhs1p (Craven *et al.*, 1996). SRP and its receptor, docking protein, are required for the co-translational targeting reaction in yeast as well as in mammalian cells. For the translocation reaction, i.e. protein insertion into the membrane and completion of transport, a requirement of BiP has been demonstrated for yeast microsomes but not for mammalian microsomes. However, lumenal proteins were found to be necessary for efficient translocation of RNP-dependent precursor proteins into mammalian microsomes (Nicchitta and Blobel, 1993).

Photoaffinity labelling experiments using azido-ATP (Klappa *et al.*, 1991; Zimmerman and Walter, 1991; Zimmermann *et al.*, 1991) led to the conclusion that an ATP-dependent component must be involved in the process of insertion of both RNP-dependent and -independent transport substrates into membranes of mammalian microsomes. On the one hand, we observed photoinactivation of transport of a chemically synthesized precursor that is translocated independently of RNP and molecular chaperones. On the other hand, when studying the transport of RNP-dependent preprolactin a similar sensitivity to azido-ATP was found that was confined to protease-resistant binding of the nascent polypeptide to microsomal membranes and its association with the TRAM–Sec61 complex. These effects were not a result of BiP-inactivation, since depleting microsomes of BiP by 90% did not result in any inhibition of transport or binding (Klappa *et al.*, 1991). Thus, the azido-ATP-sensitive component acts immediately before the translocase. Other laboratories (Müsch *et al.*, 1992; Sanders *et al.*, 1992) observed that translocation intermediates in yeast microsomes require an ATP-dependent component to become associated with the Sec61 protein.

The biochemical approach

An assay that permits screening for such a component was developed, based on reconstitution of a microsomal detergent extract into proteoliposomes (Figure 1). Upon fractionation of the detergent extract by affinity chromatography on ATP–agarose (Figure 3A) we tested proteoliposomes derived from different fractions for transport of preprolactin (Figures 2 and 4) and also for productive binding, i.e. for association of nascent preprolactin with the TRAM–Sec61 complex (Figure 5). Using M13 procoat protein, which assembles into the membrane independently of the azido-ATP-sensitive component (Table I), we were able to discriminate specific from non-specific effects (Figure 2).

The results obtained indicate that depleting a microsomal extract of ATP-binding proteins by passage through ATP–agarose leads to a pronounced reduction of transport of preprolactin. Re-addition of the ATP-eluate restored the reconstituted transport activity to control levels (Figures 2, 4 and 5). Functional analysis of proteoliposomes which were made from the pass-through of ATP–agarose revealed a phenotype that is summarized in Table I. This phenotype was the same as previously found for microsomes that were photoaffinity labelled with azido-ATP under conditions that exclude docking protein as a potential target of inactivation, i.e. by using trypsinized microsomes that after treatment with azido-ATP were assayed in the presence of the elastase fragment of docking protein (Table I). In contrast to the photolabelling approach, however, we could now reverse this phenotype by co-reconstituting the ATP-eluate. In conclusion, this eluate must contain the ATP-requiring component searched for.

Characterization of the ATP-binding proteins by various methods revealed that the majority of proteins found in the ATP-eluate were lumenal proteins. We identified BiP (ABP78), calreticulin (ABP57), Grp94 (ABP103) and PDI (ABP52), four proteins that are major constituents of microsomes and in one way or another are involved in protein folding inside the ER (Lambert and Freedman, 1983; Pelham, 1986; Ellis, 1987; Rothman, 1989; Gething and Sambrook, 1992; Dierks *et al.*, 1993). Furthermore, we identified Grp170 (ABP150) which is ~10-fold less abundant as compared with these other lumenal proteins

and which may also be involved in protein folding (Lin *et al.*, 1993).

The only membrane protein identified in the ATP-eluate is ribophorin I. This protein was described to function in the oligosaccharide transferase reaction (Crimaudo *et al.*, 1987; Kelleher *et al.*, 1992). Ribophorin I contains a lumenal β-phosphate-binding cassette which, however, is truncated as compared with those of BiP, calreticulin and Grp170. Considering furthermore the ATP-binding sequences of Grp94 (see Results), for three out of eight proteins, namely PDI and the two ribosomal proteins, there is no obvious indication of a direct interaction with ATP. As noted above, the appearance of PDI in ATP-eluate may have been due to its ability to interact with calreticulin (Baksh *et al.*, 1995).

It has to be noted that the degree of depletion by passage through ATP–agarose varied considerably between the different ATP-binding proteins. Nearly quantitative depletion was observed for BiP and strong depletion for Grp170. These proteins therefore were selected as primary candidates that could be responsible for transport activation. However, it must also be taken into consideration that a combination of two proteins present in the ATP-eluate could be involved.

Is there a role for BiP?

A direct participation of BiP in protein translocation into the ER was first proposed by Vogel *et al.* (1990), by Nguyen *et al.* (1991), and by Schekman and co-workers (Sanders *et al.*, 1992; Brodsky *et al.*, 1993, 1995). BiP obviously interacts functionally with two membrane proteins in yeast. On the one hand, Sec63 protein is supposed to function as a BiP receptor due to its DnaJ domain (Toyn *et al.*, 1988; Rothblatt *et al.*, 1989; Sadler *et al.*, 1989; Feldheim *et al.*, 1992). On the other hand, the ATP carrier is necessary to supply BiP with its co-substrate ATP (Mayinger and Meyer, 1993; Mayinger *et al.*, 1995).

Investigating yeast microsomes, it was possible to crosslink BiP with a translocating polypeptide intermediate. Temperature-sensitive mutations in the BiP-encoding gene led to translocation defects both in microsomes and in proteoliposomes derived from these mutants. Surprisingly, in two of these mutants an early step, namely the association of the translocating polypeptide with Sec61 protein, was affected, implying that there is a *trans*-effect of lumenal BiP on protein insertion at the cytosolic membrane surface. In contrast to these data obtained with yeast microsomes, neither an association of BiP with a translocating polypeptide chain nor a direct role of BiP in protein transport could be demonstrated for mammalian microsomes (Bulleid and Freedman, 1988; Zimmerman and Walter, 1990; Klappa *et al.*, 1991). The present study again argues against BiP as the crucial ATP-binding protein searched for.

Grp170 as a component of the translocase?

In yeast, the same phenotype as described above for BiP mutants was observed for Lhs1p mutants (Craven *et al.*, 1996) and there is genetic evidence that Lhs1 protein and Sec63 protein also function together in a complex (R.A.Craven and C.J.Stirling, personal communication) facilitating interaction of Sec61 protein with the translocating polypeptide. Here, we observed in our *in vitro* system a phenotype for the ATP-eluate that would be expected for a mammalian Lhs1p homologue. Furthermore, sequence comparison of Lhs1p and Grp170 showed a remarkable degree of similarity. Overall we detected 45.83% similarity (21.36% identity); for the peptide-binding domain the similarity was 35.68% (13.73% identity). Both proteins were previously identified as members of the Hsp70 family (Chen *et al.*, 1996; Craven *et al.*, 1996). Because of this similarity between Grp170 and Lhs1p and since Grp170 and BiP (see above) appeared as the only candidate proteins which could have been responsible for the negative effect of depletion of ATP-binding proteins and the positive effect of the ATP-eluate, we suggest that Grp170 may be involved in protein transport into mammalian microsomes. However, proof for this suggestion can come only from purification of Grp170 to homogeneity and successful reconstitution of the purified protein with the ATP–agarose pass-through.

A further point must be considered. In order to become reconstituted into proteoliposomes, on the one hand, and to function in our system in analogy to Lhs1p and BiP, in yeast, on the other hand, Grp170 would have to associate with a membrane protein, most likely a homologue of Sec63 protein, during reconstitution. We note that a mammalian Sec63 protein homologue was recently discovered in various mouse tissues (Brightman *et al.*, 1995) and that minute amounts of Grp170 can be detected in control proteoliposomes (data not shown).

Mechanistic considerations

The common view of protein transport into the ER is based on the analysis mainly of co-translational transport into mammalian microsomes and post-translational transport into yeast microsomes. It can be summarized that there is a co-translational mechanism where the ribosome pushes the polypeptide in transit through the translocase and that there is a post-translational mechanism where BiP pulls the polypeptide in transit through the translocase.

The following model was proposed for post-translational protein transport into yeast microsomes (Lyman and Schekman, 1995). In the first phase, the precursor binds to the signal sequence receptor complex in an ATP-independent manner. In the second, ATP-dependent phase, BiP (Kar2 protein) and Sec63 protein mediate the transfer of the precursor from the signal sequence receptor complex to the Sec61 protein complex. In the third and final phase, BiP and Sec63 protein facilitate completion of translocation in an ATP-dependent manner. Although knowledge about the role(s) of Lhs1p in transport is limited as compared with what is known about BiP, it appears that both molecular chaperones have overlapping function(s) in transport (Craven *et al.*, 1996). There is good reason to believe that this model is also valid for co-translational transport (Brodsky *et al.*, 1995). In this case, however, after initial contact with the Sec61 protein complex (second phase) the precursor in transit may be pushed through the translocase by elongation, provided that there is continuous tight contact between the translating ribosome and the translocase.

The data on co-translational transport into mammalian microsomes presented here could easily be fitted into the framework of this model. The proteins which are present in the ATP-eluate (i.e. BiP or/and Grp170) showed exactly

the phenotype which would be expected for yeast BiP or Lhs1p in a similar experimental set-up. They were required for efficient transport and appeared to be involved in interaction of the precursor with the Sec61 protein complex. However, these data appear to contradict the observation that protein transport into mammalian microsomes can be reconstituted in the absence of lumenal proteins (Görlich and Rapoport, 1993). Two observations have to be taken into account in this context. Nicchitta and Blobel (1993) have already provided evidence that lumenal components are necessary for net transfer of proteins into the mammalian ER. Panzner *et al.* (1995) reported that post-translational protein transport into yeast microsomes can also be reconstituted in the absence of BiP. Therefore, we favour the conclusion that the model which was put forward for protein transport into yeast microsomes is also valid for mammalian microsomes.

Materials and methods

Materials

Western blotting detection reagents (ECL) and [^{35}S]methionine were purchased from Amersham-Buchler. ATP-C^{8}–agarose, concanavalin A–agarose (type III-AS), puromycin, anti-rabbit, anti-rat and anti-mouse IgG–peroxidase conjugates were obtained from Sigma, CHAPS {3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate} was from Calbiochem, CNBr from Fluka, and egg yolk phospholipids (egg phosphatide extract) from Avanti Polar Lipids. Rabbit reticulocyte lysate and proteinase K were obtained from Boehringer Mannheim, Sulfolink gel, sulfo-SMCC [succinimidyl-4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate] and SMCC from Pierce, pBluescript II KS and canine pancreas cDNA library in the lambda ZAP II vector from Stratagene. The protein assay was from Bio-Rad, dialysis membranes (Spectra/Por2, diameter 6.4 mm) from Spectrum Medical Industries, PVDF membranes (Immobilon P) from Millipore, HEPES from Serva and X-ray films (X-Omat AR) from Kodak. All antibodies used were gifts from various laboratories (see below).

Functional reconstitution of protein translocation

Rough microsomes were prepared from canine pancreas and treated with micrococcal nuclease and EDTA as described (Watts *et al.*, 1983). These microsomes were washed with 0.5 M KCl (Walter and Blobel, 1980), yielding KRM, and solubilized following a similar procedure as that of Nicchitta and Blobel (1990). Usually 37.5 equivalents of KRM, as defined by Walter and Blobel (1983), were pelleted by centrifugation in a Beckman TLA100.3 rotor for 20 min at 200 000 *g*, resuspended in 20 mM HEPES–KOH, pH 7.5, 0.2 M sucrose, 0.4 M KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 2 mM DTT, 35% (w/v) glycerol to a final concentration of 0.25–1.0 equivalents/μl and solubilized by addition of CHAPS to a final concentration of 0.65% (w/v). After 20 min on ice, 24 μg egg yolk phospholipids were added in the form of sonicated liposomes followed by incubation for another 5 min. Upon centrifugation for 20 min as above, the detergent extract was removed and, for reconstitution, was dialysed overnight at 4°C against, in total, ⩾1500 volumes of 20 mM HEPES–KOH, pH 7.5, 0.25 M sucrose, 0.4 M KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1.5 mM DTT with one change of buffer after 2.5–3 h. For reconstitution of ATP–agarose fractions, typically 50–80 μg protein of the early pass-through fractions (with or without 3–5 μg protein of the ATP-eluate) were mixed with 24 μg egg yolk phospholipids and subjected to dialysis. Control samples were diluted with ATP–agarose equilibration buffer and/or elution buffer for adjustment of reconstitution conditions. After dialysis the proteoliposomes were collected by centrifugation for 30 min (as above) and resuspended in 15 μl of 20 mM HEPES–KOH, pH 7.5, 0.2 M sucrose, 50 mM KCl, 2 mM $MgCl_2$, 2 mM DTT.

Translation/translocation assays

Bovine preprolactin and M13 procoat protein were synthesized in a rabbit reticulocyte lysate in the presence of [^{35}S]methionine (Schlenstedt *et al.*, 1990) and proteoliposomes, as is indicated in the context of each experiment. The translation reaction (6.25 μl) after 60 min at 30°C was chilled on ice and subjected to sequestration analysis. For this purpose one aliquot was incubated further in the absence of protease, another aliquot in the presence of proteinase K at a final concentration of 50 μg/ml (in the case of preprolactin) or 175 μg/ml (in the case of M 13 procoat protein) for 60 min at 0°C. Where indicated, Triton X-100 was also added to a final concentration of 0.1%. Proteolysis was stopped by phenylmethylsulfonyl fluoride (10 mM). Finally, samples were boiled in sample buffer (Laemmli, 1970) and subjected to electrophoresis in high Tris–urea/SDS–polyacrylamide gels (19.5%) according to Schlenstedt *et al.* (1990). For fluorography, the gels were treated with 1 M sodium salicylate (Chamberlain, 1979). Densitometric analysis of X-ray films was performed with an LKB Ultroscan XL laser densitometer.

Chromatography on ATP–agarose

A microsomal detergent extract, prepared from 1000 equivalents KRM at 1 equivalent/μl, was applied on ATP–agarose, which was packed into a 10×100 mm column and equilibrated with 20 mM HEPES–KOH, pH 7.5, 0.4 M KCl, 1.5 mM $MgCl_2$ (or 10 mM $MnCl_2$), 1 mM EDTA, 2 mM DTT, 30% (w/v) glycerol, 0.65% (w/v) CHAPS. Following collection of the pass-through fractions the column was washed first with at least one column volume of equilibration buffer (high-salt wash) and second with three column volumes of 20 mM HEPES–KOH, pH 7.5, 4 mM $MgCl_2$ (or 10 mM $MnCl_2$), 1 mM EDTA, 2 mM DTT, 30% (w/v) glycerol, 0.65% (w/v) CHAPS (low-salt wash). Bound proteins were eluted in steps of 4 mM Mg-ATP in equilibration buffer and 10 mM Mg-ATP in equilibration buffer containing 2 M KCl but no glycerol. The fraction eluting with 4 mM ATP is referred to as ATP-eluate. Whether $MgCl_2$ or $MnCl_2$ was used is indicated in the respective legends to the figures. Purification of BiP from bovine pancreas was carried out on the same affinity medium according to the protocol of Wiech *et al.* (1993).

Characterization of ATP-binding proteins

Protein was determined as described by Bradford (1976) using the Bio-Rad protein assay according to the manufacturer's instructions (microassay procedure).

For SDS–PAGE of detergent extracts, liposomes and ATP–agarose fractions the method of Laemmli (1970) was modified using 10% acrylamide/0.13% bis-acrylamide and 0.75 M Tris–HCl, pH 8.8, for the separation gel.

For silver staining of SDS gels, the basic protocol of Wray *et al.* (1981) was followed. Fixation, however, was carried out without $CuCl_2$ and the staining step was preceded by an incubation for 10 min with 50% methanol, 0.037% formaldehyde and subsequent washing with water.

For Western blot analysis proteins were separated by SDS gel electrophoresis (as above) and transferred to PVDF membranes (Immobilon P) by semi-dry blotting in 20 mM Tris, 150 mM glycine, 20% methanol at 1 mA/cm^2 for 60 min. The membranes were blocked with 5% (w/v) milk powder in Tris-buffered saline (TBS) for 60 min and immunodecorated by (i) incubation with the respective antibody, as indicated in the legends, (ii) washing with TBS, 0.05% Triton X-100 and with TBS, (iii) incubation with the corresponding secondary antibody–peroxidase conjugate. After another washing step, detection of bound antibody was performed by coupled chemiluminescence (ECL, Amersham) according to the manufacturer's instructions. Chemiluminescence was quantitated by densitometric analysis of X-ray films, exposed for various times, with a LKB Ultroscan XL laser densitometer. The following primary antibodies were used: rabbit anti-TRAM protein (gift of Tom Rapoport, Boston), rabbit anti-sec61α protein (T.R.), rabbit anti-signal peptidase (T.R.), rabbit anti-docking protein (α-subunit) (David Meyer, Los Angeles), rabbit anti-ERp180 (D.M.), mouse anti-ribophorin I (D.M.), rabbit anti-docking protein (β-subunit) (Peter Walter, San Francisco), rat anti-Hsp70, which also recognizes BiP (Susan Lindquist, Chicago), rabbit anti-calreticulin (H.-D.Söling, Göttingen) and rabbit anti-KDEL (H.-D.S.). For subsequent immunodecorations with different antibodies, old antibodies were stripped off the blot after each decoration cycle by incubation with TBS, 0.05% Triton X-100, 10 mM DTT for at least 45 min.

For amino-terminal protein sequencing the ATP-eluate of a large-scale chromatography, starting with 4000 equivalents of KRM, was separated by SDS gel electrophoresis and electroblotted on PVDF membranes as above. The Coomassie-stained bands (ABP150, ABP78) were cut out and subjected to Edman degradation on a 477A Protein Sequencer (Applied Biosystems). For sequencing of ABP67 and ABP57, 4 ml of the ATP-eluate, before electrophoresis, was precipitated according to Wessel and Flügge (1984).

BrCN fragmentation of ABP37, ABP45, ABP52 and ABP103 was carried out as follows. Up to 10 ml of ATP-eluate were separated on SDS gels (see above) and the Coomassie-stained bands were cut into gel slices, which were dried *in vacuo* and subjected to *in situ* BrCN cleavage according to Jahnen *et al.* (1990). The gel slices were dried again, equilibrated with 1 M Tris and loaded on a 17.5% acrylamide/0.23% bis-acrylamide high-Tris/SDS gel (cf. above). Following electrophoresis and transfer to PVDF membranes, amino acid sequencing of the peptides was performed as described above.

Chromatography on Concanavalin A–agarose was carried out as reported by Görlich *et al.* (1992a) using CHAPS in place of digitonin.

For phase-separation with Triton X-114 we followed a protocol based on those of Bordier (1981) and of Tiruppathi *et al.* (1986), introducing minor modifications: 40 µl proteoliposomes (or microsomes) were treated with 400 µl of 200 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% (w/v) Triton X-114 and incubated on ice for 20 min. Unsolubilized material was removed by centrifugation in the Beckman TLA100.3 rotor at 100 000 *g* for 60 min. The supernatant was layered on 300 µl of a cushion containing 200 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.06% (w/v) Triton X-114, 6% (w/v) sucrose and incubated at 30°C for 5 min. The cloudy solution was centrifuged for 3 min at 6000 *g* in an Eppendorf centrifuge at room temperature to separate the detergent-poor phase (DPP) from the detergent-rich phase (DRP). DPP, containing hydrophilic proteins, was washed by adjustment of the Triton X-114 concentration to 0.5%, incubation for 5 min and centrifugation as above. DRP, containing hydrophobic proteins, was separated from contaminating DPP by dilution with 1 ml of 200 mM Tris–HCl, pH 7.4, 150 mM NaCl, incubation at 0°C for 10 min and at 30°C for 5 min and centrifugation as above.

Antibodies directed against ABP150 and ABP45 were generated in rabbits by synthesizing the following peptides: LAVMSVDLGSEC and LEAKSEEKGC. The peptides were coupled to keyhole limpet haemocyanin, activated with sulfo-SMCC. Where indicated antibodies were affinity-purified on Sulfo-link gel with coupled peptides.

cDNA cloning and sequence analysis

The cDNA which is coding for ABP45 was isolated by screening primary plaques of a canine pancreas cDNA library in the lambda ZAP II vector with affinity-purified antibodies against ABP45. Positive clones were identified and inserts were isolated using appropriate restriction enzymes and subcloned into pBluescript IIKS. The sequence is available under accession number X99909.

Sequence comparison for proteins Grp170 and Lhs1p was carried out following the Clustal method, provided by the DNASTAR sequence analysis software.

Crosslinking experiments

Nascent preprolactin (ppl-86mer) was synthesized by *in vitro* transcription from a *Pvu*II-linearized plasmid coding for bovine preprolactin (Zimmermann *et al.*, 1991) followed by translation in a reticulocyte lysate at 30°C for 20 min in the presence of [^{35}S]methionine and proteoliposomes (2 µl per 18.75 µl translation reaction). Samples were chilled on ice and centrifuged at 200 000 *g* at 2°C for 60 min. Pelleted membranes were resuspended in 50 mM HEPES–KOH, pH 7.5, 50 mM potassium acetate, 2 mM magnesium acetate, 0.2 M sucrose. Aliquots were frozen in liquid nitrogen either directly or after 4 or 20 min of incubation with puromycin (1.25 mM) at 30°C (puromycin chase). Chemical crosslinking of the thawed samples was carried out on ice for 30 min adding succinimidyl-4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate to a final concentration of 75 µg/ml. Analysis of translation and crosslink products by SDS gel electrophoresis and fluorography was performed as described for the translation/translocation assay.

Acknowledgements

We thank Drs S.Lindquist (Chicago), D.Meyer (Los Angeles), T.Rapoport (Boston), H.-D.Söling (Göttingen), J.Stahl (Berlin) and P.Walter (San Francisco) for gifts of antibodies. This work was supported by the 'Deutsche Forschungsgemeinschaft' and by the 'Fonds der Chemischen Industrie'.

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Received on July 12, 1996; revised on September 16, 1996