Identification of signal sequence binding proteins integrated into the rough endoplasmic reticulum membrane

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An azidophenacyl derivative of a chemically synthesized consensus signal peptide has been prepared. The peptide, when photoactivated in the presence of rough or high-salt-stripped microsomes from pancreas, leads to inhibition of their activity in cotranslational processing of secretory pre-proteins translated from their mRNA *in vitro*. The peptide binds specifically with high affinity to components in the microsomal membranes from pancreas and liver, and photoreaction of a radioactive form of the azidophenacyl derivative leads to covalent linkage to yield two closely related radiolabelled proteins of M_r about 45000. These proteins are integrated into the membrane, with large 30000- M_r domains embedded into the phospholipid bilayer to which the signal peptide binds. A smaller, endopeptidase-sensitive, domain is exposed on the cytoplasmic surface of the microsomal vesicles. The specificity and selectivity of the binding of azidophenacyl-derivatized consensus signal peptide was demonstrated by concentration-dependent inhibition of photolabelling by the 'cold' synthetic consensus signal peptide and by a natural internal signal sequence cleaved and isolated from ovalbumin. The properties of the labelled 45000- M_r protein–signal peptide complexes, i.e. mass, pI, ease of dissociation from the membrane by detergent or salts and immunological properties, distinguish them from other proteins, e.g. subunits of signal recognition particle, docking protein and signal peptidase, already known to be involved in targetting and processing of nascent secretory proteins at the rough endoplasmic reticulum membrane. Although the 45000- M_r signal peptide binding protein displays properties similar to those of the signal peptidase, a component of the endoplasmic reticulum, the azido-derivatized consensus signal peptide does not interact with it. It is proposed that the endoplasmic reticulum proteins with which the azidophenacyl-derivatized consensus signal peptide interacts to yield the 45000- M_r adducts may act as receptors for signals in nascent secretory pre-proteins in transduction of changes in the endoplasmic reticulum which bring about translocation of secretory protein across the membrane.

INTRODUCTION

Many proteins destined for export or localization into lysosomal and plasma membranes are initially made on cytoplasmic ribosomes with hydrophobic *N*-terminal extensions called signal sequences (Walter *et al.*, 1984). Signal sequences are thought to function, at least in eukaryotic cells, by ensuring that their polysomes are directed to the endoplasmic reticulum to form a functional ribosome–membrane junction culminating in translocation of the growing polypeptide chain across the endoplasmic reticulum. In most cases, signal sequences are removed either during or immediately after segregation into the endoplasmic reticulum cisternae by lumenally-localized signal peptidase (Jackson & Blobel, 1980).

The components of the molecular machinery that guide nascent polypeptides containing signal sequences from cytosol to the endoplasmic reticulum membrane have been identified. They include the signal recognition particle (SRP) which consists of six distinct polypeptides and one 7 S RNA molecule (Walter & Blobel, 1982)

which binds with high affinity to polysomes translating proteins carrying signal sequences, imposing a transient arrest of polypeptide chain elongation. This interaction occurs between the signal sequence of nascent polypeptide emerging from the ribosome and the 54000- M_r subunit of the SRP (Kurzchalia *et al.*, 1986). The translation arrest is overcome by direct interaction of SRP with its receptor, docking protein (Meyer *et al.*, 1982), an integral cytoplasmically-disposed protein of the endoplasmic reticulum membrane. Binding between SRP and docking protein is a transient event causing dissociation of the former from the ribosomal complex and is not directly involved in the maintenance of the ribosome–membrane junction (Gilmore & Blobel, 1983).

In contrast with our present understanding of SRP–docking protein interactions, little is known about the subsequent mechanisms by which signal sequence integrates with and penetrates into the membrane phase, or the events that lead to translocation of the preceding polypeptide chain across the endoplasmic reticulum membrane. It is not known whether translocation is directly through lipid or through a proteinaceous pore or

Abbreviations used: ACSP, Cys¹²-*S*-(*p*-azidophenacyl)-consensus signal peptide; SDS/PAGE, SDS/polyacrylamide-gel electrophoresis; SRP, signal recognition particle.

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how transient formation of such a pore would ensue. Nevertheless, there is genetic (Ferro-Novick, 1985) and biochemical (Gilmore & Blobel, 1985) evidence that there are other, yet unidentified, proteins that are involved in stages after interactions with SRP and docking protein. It is known, for example, that microsomal membrane vesicles when extensively proteolysed are functionally inactive in segregating and processing pre-proteins *in vitro*, even when reconstituted with the 60000- M_r fragment of the docking protein (Gilmore *et al.*, 1982).

In our previous publication (Austen *et al.*, 1984) we reported the chemical synthesis of a model consensus signal peptide, the design of which was based on common structural features found in natural signal sequences. The peptide was demonstrated to inhibit in a dose-dependent manner the processing and segregation of several distinct secretory pre-proteins biosynthesized from their mRNAs in a cell-free translation system supplemented with dog pancreatic microsomal vesicles. Inhibition occurred at the cytoplasmic surface of the microsomal vesicles, resulting in accumulation of the precursors which were shown to be susceptible to exogenously added proteinases. Thus, it was of interest to understand the mode of interaction of signal peptides with the microsomal membranes and identify the site of action. Towards this goal, we have converted the consensus signal peptide into a radioactive, photoactivatable azido derivative which, when activated during incubations with microsomal membranes from tissues highly active in protein secretion, leads to selective and specific binding and labelling of a closely-related pair of novel 45000- M_r proteins integrated into the endoplasmic reticulum membrane. The paper describes identification of the 45000- M_r signal peptide binding proteins and some of their properties in relation to SRP, docking protein and signal peptidase.

EXPERIMENTAL PROCEDURES

Chemicals

All chemicals, unless stated otherwise, were purchased from Sigma and BDH.

Peptides

The synthesis, purification and characterization of the 19-residue consensus signal peptide has been described (Austen *et al.*, 1984). A natural signal peptide which comprised residues 21–47 of ovalbumin was isolated by h.p.l.c. after tryptic digestion of *S*-sulphonylated ovalbumin (Robinson *et al.*, 1986). A fragment of human parathyroid hormone (residues 28–48) and γ -endorphin were purchased from Cambridge Research Biochemicals (Cambridge, U.K.).

Preparation of an azido derivative of the consensus signal peptide and its radioiodination

The following operations were carried out in the dark. The reduced form of the 19-residue consensus peptide dissolved in a final volume of 1 ml of 0.1 M-Tris/HCl buffer, pH 7.2, at a concentration of 0.26 mM, was treated with a 1.8-fold molar excess of *p*-azidophenacyl bromide (Pierce and Warriner Ltd.) for 1 h at 22 °C. The extent of the reaction involving alkylation between the free thiol group of cysteine (residue 12 of the peptide) and *p*-azidophenacyl bromide was monitored by the reagent

5,5'-dithiobis-(2-nitrobenzoic acid) as an indicator of surviving free thiol groups (Ellman, 1959) with respect to time. By 1 h, the reaction had reached completion. The product, Cys¹²-*S*-(*p*-azidophenacyl)-signal peptide (ACSP) was purified by chromatography on Sephadex G-25 in 30% (v/v) acetic acid. The modified peptide was characterized by determining its amino acid composition after hydrolysis in 6 M-HCl at 110 °C for 20 h, and by analysis of its absorbance spectrum in the u.v. region (see the Results section).

For radioiodination, 2.5 nmol of ACSP dissolved in 20 μ l of 0.3 M-phosphate buffer, pH 7.4, was mixed with 1.5 mCi of carrier-free ¹²⁵I (Amersham International) in the presence of 48 μ g of chloramine-T for 30 s. After desalting on a Sephadex G-25 column (1 cm internal diameter $\times$ 30 cm) in 30% (v/v) acetic acid, ACSP was assessed to be radiolabelled to a specific activity of 2.33×10^8 c.p.m./nmol. The radioiodinated ACSP (¹²⁵I-ACSP) was freed of acetic acid by rotary evaporation before redissolving in a suitable volume of 50 mM-Hepes/KOH buffer, pH 7.4, prior to its application in incubations with membranes or other experiments described below.

Preparation of rough and stripped microsomal membranes

Rough microsomal membrane vesicles were prepared as described by Kaderbhai & Austen (1984) from pancreas of dogs fed *ad libitum*. The animals were preadministered with 2 dog units of pancreozymin/cholecystokinin (Boots, Nottingham, U.K.)/kg body wt., 40 min before killing and excision of the pancreas. Membranes depleted of ribosomes (stripped microsomal membranes) were prepared by gently mixing freshly-prepared rough microsomal membranes suspended in 1.65 M-sucrose/25 mM-KCl/5 mM-MgCl₂/50 mM-Tris/HCl, pH 7.4, with 3 vol. of 0.7 M-KCl/15 mM-EDTA/25 mM-Hepes/KOH, pH 7.4, at 0 °C, and then incubating at 0 °C for 20 min. The salt-treated membranes were layered over a 0.75 M-sucrose cushion containing 0.5 M-KCl and 20 mM-Hepes/KOH, pH 7.4, and centrifuged at 105000 g_{av} for 1.5 h at 4 °C. The resulting stripped membrane vesicles were harvested as a suspension from the sucrose cushion, diluted 3-fold with 20 mM-Hepes/KOH, pH 7.4, re-centrifuged as above, and the pellet derived was gently homogenized in 0.25 M-sucrose/20 mM-Hepes/KOH, pH 7.4.

Rat liver microsomal membranes were prepared by the method described by Sandberg *et al.* (1980), and portions were treated with 0.1 M-Na₂CO₃ (Fujiki *et al.*, 1982). Rat liver mitochondria were prepared as follows. Rat liver homogenate (1 g/2 ml) in 0.25 M-sucrose/10 mM-Tris/HCl, pH 7.4, was centrifuged at 1000 g_{av} for 10 min at 4 °C to obtain a post-nuclear supernatant from which a crude mitochondrial pellet was obtained by centrifugation at 10000 g_{av} for 20 min at 4 °C. The membranous pellet was suspended in 1.54 M-sucrose/10 mM-Tris/HCl, pH 7.4, and freed from Golgi membranes by centrifugation through a discontinuous sucrose gradient as described by Fleisher & Fleisher (1970).

Cell-free translation system

Procedures for isolation of poly(A)-rich bovine pituitary mRNAs, their translation in reticulocyte lysates supplemented with micrococcal nuclease-treated rough or stripped microsomal membrane vesicles, and

the analysis of the products translated *in vitro* by separation in 15% (w/v) polyacrylamide gels in the presence of SDS and fluorography have been described by Kaderbhai & Austen (1985).

Incubation conditions for photoactivation of ^{125}I -ACSP with microsomal membranes

Unless stated otherwise, microsomal membranes suspended at protein concentrations of 6–8.6 mg/ml were incubated with 200 nM- ^{125}I -ACSP in a total volume of 100 μl of N_2 -flushed 0.25 M-sucrose/70 mM-potassium acetate/3.5 mM-magnesium acetate/50 mM-Hepes/KOH, pH 7.4 (SHMK buffer), contained in a sealed Pyrex tube, and irradiated from a distance of 9 cm with light emitted in a region greater than 300 nm wavelength from a multiband u.v. light torch, model SL-58 50 Hz (Ultraviolet Products, San Gabriel, CA, U.S.A.), for 2 h at 4 °C. In some experiments where stated, the radiolabelled microsomal membranes were then washed by diluting the reaction mixture with 9 vol. of chilled 50 mM-Hepes/KOH buffer, pH 7.4, centrifuged at 12000 g_{av} for 15 min and the recovered pellets prepared for electrophoretic analysis.

Photoactivatable modification of microsomal membrane phospholipids by ^{125}I -ACSP

Washed, ^{125}I -ACSP modified microsomal membrane vesicles containing 750 μg of protein were suspended in 100 μl of water. The membranes were extracted three times with 1 ml of chloroform/methanol (2:1, v/v). The pooled chloroform extracts were re-extracted with 0.2 ml of water, yielding an organic top layer and an aqueous lower layer. The amount of phospholipid recovered in the organic phase was determined by the method of Hess & Derr (1975), and the radioactivity associated with this fraction was determined by γ -counting.

Signal peptidase preparation and assay

(i) A preparation of signal peptidase from rat liver was partially purified as previously described (Mollay *et al.*, 1982) and was kindly provided by Dr. C. Mollay, Institute for Molecular Biology, Austrian Academy of Sciences, Salzburg, Austria. This preparation (3 mg of protein) was reconstituted with 4 mg of phosphatidylethanolamine in a final 1 ml volume containing 0.25% (w/v) sodium deoxycholate, 0.5% (w/v) sodium cholate and 10 mM-Tris/HCl, pH 7.4, by dialysis against 10 mM-Tris/HCl, pH 7.4, at 4 °C for 70 h. Liposomes were collected by centrifugation at 100000 g_{av} for 2 h at 4 °C to give a preparation containing 0.35 mg of protein and 17 μmol of phosphorus in 1 ml. Signal peptidase assays were performed using 10 μl of wheat germ cell-free translation system containing [^3H]pre-promellitin synthesized *in vitro* (10000 c.p.m.) to which either reconstituted vesicles (9 μg of protein) or alkali-stripped rough microsomes (50 μg of protein) was added in a final 100 μl volume buffered with 0.1 M-Tris/HCl, pH 8; the mixture was incubated for 2 h at 25 °C. The extent of signal peptide cleavage was determined from the amount of ^3H associated with peptides isolated by paper electrophoresis and chromatography (Mollay *et al.*, 1982). Since the signal peptidase activity under the above assay conditions is linear only up to 40% of the substrate conversion with the enzyme concentration, the assays were performed under conditions which gave about 30% processing of pre-promellitin (Mollay *et al.*, 1982).

(ii) A volume (10 μl) of 10 or 25 mM-octyl glucoside-solubilized extracts of dog pancreatic rough microsomal protein (3 mg/ml) in 2.5 mM-magnesium acetate/5 mM-dithiothreitol/25 mM-Hepes/KOH, pH 7.4, was added to 2 μl of post-translational reticulocyte lysate mixture containing [^{35}S]preprolactin synthesized *in vitro* (110000 c.p.m.) and incubated at 25 °C for 1 h. The extent of pre-prolactin conversion to prolactin was monitored by densitometry of the fluorographs of the products separated on 15% (w/v) polyacrylamide gels in SDS. One unit of the signal peptidase activity is defined as the amount of enzyme (protein) that gave 10% conversion of the precursor protein under the standard conditions.

Electrophoretic separation of proteins

SDS/PAGE. For routine analysis of microsomal membrane proteins by SDS/PAGE, the samples were prepared by mixing with an equal volume of SDS sample buffer composed of 10% (w/v) SDS, 30% (w/v) sucrose, 50 mM-dithiothreitol, 0.003% Bromophenol Blue and 10 mM-Tris/HCl, pH 6.8, and then boiling for 3 min. After cooling to room temperature, a volume of the sample containing 100–250 μg of protein was loaded in each well of slab gels consisting of a stacking gel over a linear polyacrylamide gradient ranging from 7 to 16% in the running gel system containing 0.1% (w/v) SDS (Neville, 1971). The proteins were resolved by applying a fixed current of 10 mA under a variable voltage field (max. 250 V). The resolved proteins were detected by staining with Coomassie Blue R-250, or in the case of radiolabelled proteins by standard autoradiography of the dried gel.

The electrophoretic conditions to separate ^{35}S -labelled proteins in rabbit reticulocyte translation systems were essentially as described above except that the samples were prepared by mixing with 3 vol. of SDS sample buffer and resolved in 15% (w/v) homogeneous polyacrylamide gels and the products detected by fluorography of the dried gels.

Two-dimensional gel electrophoresis. This was performed as described previously (Kaderbhai & Austen, 1984).

Western transfer and immunoblotting

Total microsomal membrane protein fractions were resolved in two-dimensional isoelectrofocusing-SDS/PAGE gels and then electrotransferred onto nitrocellulose sheets. The identity and position of the docking protein was established by a mouse anti-(docking protein) monoclonal antibody (a gift from Dr. D. Meyer, EMBL, Heidelberg, Germany) and rabbit anti-(mouse IgG) coupled to peroxidase (Batteiger *et al.*, 1982).

RESULTS

Preparation and properties of photoactivatable ACSP

The consensus signal peptide, Lys-Lys-Ser-Ala-Leu-Leu-Ala-Leu-Met-Tyr-Val-Cys-Pro-Gly-Lys-Ala-Asp-Lys-Glu, reduced from its *S*-sulphonylated form by dithiothreitol to generate the cysteinyl thiol group (Austen *et al.*, 1984) was alkylated with the photoactivatable reagent *p*-azidophenacyl bromide as described in the Experimental procedures section. The stoichiometry of the reaction between the consensus peptide and *p*-azidophenacyl bromide was deduced to be 1:1 by

Fig. 1. Structure of ACSP (a) and its photoactivation and decay as a function of time (b)

(a) The synthesis, purification and reduction of the consensus signal peptide was as described previously (Austen *et al.*, 1984). [³H]Leucine was incorporated at position 6 from the *N*-terminus to monitor purification of the peptide after solid-phase synthesis. *S*-Alkylation of cysteine at position 12 with *p*-azidophenacyl bromide was performed in the dark as described in the Experimental procedures section. Afterwards, the photoactivatable peptide was radioiodinated at Tyr-10 to a specific activity of 2.33×10^8 c.p.m./nmol. (b) ACSP ($9 \mu\text{M}$) dissolved in 50 mM-Hepes/KOH, pH 7.5, buffer was irradiated with u.v. light above 300 nm as described in the Experimental procedures section. Samples were withdrawn at 0 min (1), 30 min (2), 80 min (3) and 150 min (4), and their absorbance spectra were monitored between 250 and 350 nm. ACSP shows an absorbance maximum at 290 nm and is converted to a nitrene, with loss of absorbance, at a rate with half-life of 45 min.

determining the amino acid composition and absorbance at 300 nm of the purified product (Fig. 1a) after Sephadex G-25 chromatography, and by using the published data on the absorption coefficient, $2 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (Hixson & Hixson, 1975). The effect of irradiating ACSP for varying durations with light at wavelength greater than 300 nm on its absorption spectrum is shown in Fig. 1(b). The spectrum before irradiation exhibited an absorption maximum at 290 nm, and decayed exponentially with respect to time upon photoactivation, with a half-life of about 45 min.

Effect of ACSP on cotranslational processing

The effects of ACSP on the ability of pancreatic microsomal membranes to cotranslationally process pre-proteins in a cell-free translation system were examined in this study to check whether the alkylation with the photoactivatable reagent had affected the properties of the consensus signal peptide reported previously (Austen *et al.*, 1984). Microsomal membrane vesicles incubated with or without ACSP were irradiated with light in the u.v. region. The membranes were then added to a reticulocyte lysate translation system containing [³⁵S]methionine, immediately followed by the addition of bovine pituitary mRNA, and translation was initiated by warming to 30 °C. The results of the experiment are shown in Fig. 2, which demonstrate that ACSP, at $29 \mu\text{M}$, considerably reduced the capacity of the membranes to co-translationally process [³⁵S]pre-prolactin to prolactin (track 4). In contrast with this, irradiating the membranes in the absence of ACSP under identical conditions did not result in any observable inhibition of the processing activity in the translation system *in vitro* (track 3).

Photomediated covalent labelling of microsomal membrane proteins by ¹²⁵I-ACSP

In order to investigate further the nature and site of action of ACSP in the microsomal membranes, ACSP

Fig. 2. Inhibition of co-translational processing of pre-prolactin by ACSP

Nuclease-treated dog pancreatic rough microsomal vesicles (RM_n), $47 A_{260}$ units/ml, were incubated with (track 4) or without (track 3) ACSP at 0.3 mM in 0.25 M-sucrose/20 mM-Hepes/KOH (pH 7.4) at 4 °C during u.v. irradiation for 2 h. Then an aliquot was added to a nuclease-treated rabbit reticulocyte translation system with [³⁵S]methionine, followed immediately by the addition of bovine pituitary mRNA, to give a membrane concentration of $4.5 A_{260}$ units/ml and the peptide concentration of $29 \mu\text{M}$. Translations were continued for 1 h at 30 °C. ³⁵S-labelled proteins were separated by electrophoresis on polyacrylamide (15%) in SDS and detected by fluorography.

was radioiodinated to a high specific activity (2.33×10^8 c.p.m./nmol) and photoactivated after incubation with stripped microsomal membranes from dog pancreas. The microsomal vesicles were then washed to

Fig. 3. Labelling of pancreatic stripped microsomal membrane polypeptides after photoactivation with ^{125}I -ACSP

Stripped microsomes after incubation with ^{125}I -ACSP were photoactivated in the absence (track 1) or presence of increasing amounts of unmodified consensus signal peptide (CSP) (tracks 2–7), γ -endorphin (γ -En) (tracks 8 and 9), and a peptide comprising residues 28–48 of human parathyroid hormone (PTH) (tracks 10 and 11). After the reaction, unbound radiolabel was removed by centrifugation and the pelleted membranes dissolved in SDS sample buffer prior to their separation by SDS/PAGE. The subsequent autoradiogram of the dried gel was obtained after 3 days of exposure (tracks 1–11). Track 12 is the Coomassie Blue-stained profile of track 1, and track 13 is the profile of M_r markers.

remove excess unbound radioactivity, and the membrane constituents were resolved into organic (phospholipids) and aqueous (proteins) phases. The amount of radioactivity bound to the components in these two phases was determined. The results from five experiments showed that 92% of the total radioactivity bound to the membranes was soluble in the organic phase, the remainder being water-soluble.

Further identification of the possible target component(s) with which ^{125}I -ACSP interacted in the membrane was sought by analysis of the microsomal membrane polypeptides by SDS/PAGE. A typical electrophoretic Coomassie Blue-stained profile of polypeptides from pancreatic stripped microsomal membranes photoactivated with ^{125}I -ACSP is shown in Fig. 3, track 12. The polypeptides chemically modified by the radiolabelled azido-derivatized peptide are detectable in the corresponding autoradiogram in Fig. 3, track 1. The results show that a selective number of the membrane polypeptides with M_r in the region between 45000 and 70000 are modified, but a polypeptide of M_r 45000 was most prominently labelled. Extensive label was also incorporated into polypeptides below M_r 18000, and a radioactive substance not extracted into chloroform, which may be residual ^{125}I -ACSP not removed by centrifugation from the membranes, ran to the gel front. A careful examination of Coomassie Blue-stained polypeptide profiles of microsomal membranes photoactivated with ^{125}I -ACSP from control or untreated membranes revealed no apparent differences (results not shown), thus indicating that no observable breakdown of the proteins occurs under the condition of photoactivation used here.

Protection by signal peptide against photoactivated chemical modification of the 45000- M_r polypeptides by ^{125}I -ACSP

In order to distinguish the membrane proteins which were radiolabelled by selective and specific binding with ACSP from those labelled through non-specific interactions, photoactivation of ^{125}I -ACSP with dog pancreatic stripped-microsomal membranes was performed in the presence of increasing amounts of the unmodified consensus signal peptide (Fig. 3, tracks 2–7). To correct for unequivalent loading on the gel, for example track 3, densitometry was used to calculate the ratios of the intensities of the 45000- M_r band to the minor band at M_r 54000. The inclusion of the consensus signal peptide during photoactivation produced interesting and reproducible changes in the labelling of proteins around 45000- M_r region; formation of the 45000- M_r ^{125}I -ACSP cross-linked adduct was progressively inhibited, partially by 76% at 0.2 μM and completely at 0.5 μM . In contrast, both the pattern and the intensity of other proteins labelled were entirely unaffected by the signal peptide at all concentrations tested. Two control hydrophobic peptides, human parathyroid hormone (residues 28–48) and γ -endorphin, each tested at 1 and 10 μM , had no effect on the pattern of polypeptides labelled by ^{125}I -ACSP (Fig. 3, tracks 8–11).

Competitive inhibitory action of the ^{125}I -ACSP-mediated photoactivated labelling of 45000- M_r components was also exerted by a naturally-occurring signal sequence. An intra-chain fragment of ovalbumin (residues 21–47) bearing the signal sequence (Tabe *et al.*, 1984) was isolated and two forms of the peptide were prepared; one

Fig. 4. Inhibition of ¹²⁵I-ACSP radiolabelling of microsomal membrane proteins by ovalbumin signal peptide

Dog pancreatic stripped microsomal vesicles were u.v.-irradiated with ¹²⁵I-ACSP in the absence (tracks 1 and 8) or in the presence of increasing amounts of a purified fragment derived from residues 21–47 containing the internal signal sequence of ovalbumin in the form of the S-sulphonylated (-S-SO₃⁻) (tracks 2–4) or thiol (-SH) (tracks 5–7) peptide. The radiolabelled polypeptides were resolved and analysed as described in Fig. 3 legend.

with a S-sulphonylated cysteine residue and the other with dithiothreitol-regenerated thiol on the cysteine residue. The effect of adding varying amounts of ovalbumin signal peptides during photoactivation of ¹²⁵I-ACSP with microsomal membranes on the polypeptides' labelling are shown in Fig. 4. Both forms of the peptide at 20 μM effectively inhibited cross-linking of ¹²⁵I-ACSP to 45000-*M_r* proteins, but labelling of other minor polypeptides was unaffected (Fig. 4, tracks 4 and 7). At lower concentrations (0.2 and 2 μM) the reduced form of ovalbumin signal peptide, which would be more akin to that found in the nascent polypeptide, generated a more effective protection of labelling of the 45000-*M_r* proteins than the S-sulphonylated form (Fig. 4, compare tracks 2 and 3 with 6 and 7).

The selective covalent labelling of the 45000-*M_r* proteins after photoactivation with ¹²⁵I-ACSP was also demonstrated in rough microsomal vesicles, which had not been stripped with high salt and EDTA, from dog pancreas (Fig. 5, track 1). Compared with stripped microsomes, enhanced incorporation of the radioactivity in bands corresponding to *M_r* 59000 and 70000 was observed. It is interesting to note that modification of these polypeptides with ¹²⁵I-ACSP, unlike the 45000-*M_r* components, also occurs in the absence of photoactivation (Fig. 5, track 3). Inclusion of consensus signal peptide with rough microsomal vesicles prior to photoactivation with ¹²⁵I-ACSP inhibited the modification of the 45000-*M_r* proteins (Fig. 5, track 2) in a

Fig. 5. Labelling of rough microsomal membrane proteins from pancreas by photoactivation with ¹²⁵I-ACSP

Rough microsomes (750 μg) from dog pancreas were u.v.-irradiated with ¹²⁵I-ACSP as described in the Experimental procedures section and the legend to Fig. 3 either in the absence (track 1) or presence of 1 μM of inhibiting CSP (track 2). The effect of incubating membranes with ¹²⁵I-ACSP in the dark in the absence of u.v. light is shown in track 3. Track 4 is a control showing the lack of label associated with rat liver mitochondria (750 μg) after u.v.-irradiation with ¹²⁵I-ACSP. The membranes were solubilized with 75 mM-octyl glucoside at a protein (phospholipid) concentration of 4 mg/ml (1.4 μmol/ml) in 50 mM-Hepes/KOH, pH 7.4, containing 5 mM-magnesium acetate, 50 mM-potassium acetate and 5 mM-dithiothreitol, and by sonication applying three 15 s bursts at a power setting of 100 W (Braunsonic 1510 Sonicator) at 0 °C with 30 s cooling periods in between. The detergent-solubilized material was separated from the insoluble material by centrifugation at 440000 *g_{av}* for 4 min at 4 °C, and the soluble material was prepared for SDS/PAGE.

manner analogous to that demonstrated with stripped microsomal membranes (Fig. 3, track 6).

As a control, fresh intact mitochondria isolated from rat liver, when incubated with ¹²⁵I-ACSP, showed no significant incorporation in any of the polypeptides from this non-secretory organelle (see Fig. 5, track 4).

Properties of microsomal membrane proteins modified by ACSP

In an attempt to explore the nature of organization of the signal peptide binding proteins in the microsomal

Fig. 6. Detergent solubilization of signal peptide binding proteins

Dog pancreatic rough microsomal vesicles were u.v.-irradiated in the presence of ^{125}I -ACSP as described in the Experimental procedures sections. Aliquots were then sonicated as described in Fig. 5 legend, at a protein concentration of 4 mg/ml after solubilization with octyl glucoside at the concentrations shown, centrifuged and the derived supernatants (tracks 1–10) and pellets (tracks 11 and 12) were dissolved in SDS sample buffer and resolved by SDS/PAGE as described for Fig. 3.

membranes, rough microsomal vesicles after photoactivation with ^{125}I -ACSP were titrated with the non-ionic detergent, octyl glucoside, as described in the Experimental procedures section. The detergent-treated membranes were separated into soluble and insoluble protein fractions by ultracentrifugation, and then analysed by electrophoresis and autoradiography. The detergent-solubilized profile of the ^{125}I -ACSP-modified microsomal polypeptides (Fig. 6, tracks 1–10) revealed that the labelled 45000- M_r proteins are only released from the membrane by treatment with octyl glucoside at concentrations greater than 20 mM. In contrast, other proteins, especially those of M_r 59000 and 70000, were preferentially released from the membranes at lower concentration of the detergent. Examination of radiolabelled polypeptides remaining in the pelleted material after detergent extraction showed selective loss of 45000- M_r components at higher concentrations of octyl glucoside (Fig. 6, tracks 11 and 12).

Proteinase treatment of ^{125}I -ACSP-modified microsomal vesicles

To gain more information on the disposition of 45000- M_r proteins in microsomal membrane vesicles, freshly prepared intact pancreatic rough microsomal vesicles were treated with various proteinases after their photomediated modification with ^{125}I -ACSP. The proteinases were then inactivated and, along with the released fragments, removed by centrifugation, and then the labelled polypeptides were analysed (Fig. 7). The digestion profiles of the radiolabelled bands with the three proteinases, trypsin, elastase and chymotrypsin, each tested at three different concentrations, were similar, but the former was most efficient and the latter was least efficient. An interesting and reproducible effect of digestion is a product of M_r around 30000, the

appearance and intensity of which coincides well with the disappearance of the 45000- M_r proteins; the resulting protein remains bound to the membrane since it is not removed by washing. It would appear that the minor labelled bands of M_r 70000 and 59000 are relatively resistant to the action of the proteinases. When the digestions were performed at intermediate concentrations of the proteinases (10 $\mu\text{g}/\text{ml}$) in the presence of 1% (w/v) Triton X-100, significant reductions in the labelling ensued; it was complete with trypsin, less with elastase and little with chymotrypsin.

Possible identification of the 45000- M_r ^{125}I -ACSP-modified polypeptides

When a partially purified preparation of rat liver signal peptidase containing 6 units of activity, reconstituted into liposomes, was photoactivated with ^{125}I -ACSP, no labelling of a polypeptide in the region of M_r 45000 was observed (Figs. 8a and 8b, track 1). In contrast, rough microsomal vesicles from rat liver yielded a distinctly labelled band of around M_r 45000 (Fig. 8, track 2). The 45000- M_r protein is also labelled in the rat liver membranes containing an equivalent amount of signal peptidase activity (6 units), which had previously been washed with 0.1 M- Na_2CO_3 , a procedure known to remove ribosomes and most loosely-bound, peripheral, and luminal proteins (Fig. 8, track 3). The treatment of rough microsomal vesicles with alkali was found to reduce the protein/phospholipid (mg/ μmol) ratio from 2.3 to 0.2 in the membranes, and has been shown not to inactivate signal peptidase (Lively & Walsh, 1983). Together with this significant loss of protein from the membranes, the Coomassie Blue-stained profiles from rough and Na_2CO_3 -treated microsomal vesicles also reflect changes in their protein compositions (Fig. 8b, tracks 2 and 3). An additional important observation is

Fig. 7. Proteinase treatment of ^{125}I -ACSP-modified microsomal membranes

Dog pancreatic rough microsomal membranes after photoactivation with ^{125}I -ACSP were washed and resuspended in SHMK buffer. A stock solution of proteinases prepared at 10 mg/ml in SHMK buffer were added to the membranes to give the required final concentrations shown together with microsomal protein at 8 mg/ml in the final mixture. Digestion were normally carried out at 25 °C for 30 min, except that for the samples treated with 0.5 µg of proteinases/ml the incubation was at 0 °C for 1 h. In those digestions which required prior solubilization of the membranes, Triton X-100 was added to a final concentration of 1% (v/v). At the end of the digestion period, 100 k-i.u. of Trasylol (Bayer) were added. Those membranes not treated with the detergent were then diluted with 10 vol. of chilled 50 mM-Hepes/KOH, pH 7.4, buffer and centrifuged at 12000 $g_{av.}$ for 15 min at 4 °C. The membrane fractions and the detergent-treated digests were then separated by electrophoresis and the labelled products detected by autoradiography.

retention of radiolabel in components, in addition to the 45000- M_r components, in the region of M_r below 18000 in the Na_2CO_3 -treated membranes and reconstituted signal peptidase preparation. The fact that photoactivated modification by ^{125}I -ACSP of proteins of pancreatic microsomal membranes leads to interference of the co-translational processing activity of these vesicles (Fig. 2) led us to investigate whether this effect was due to inhibition of signal peptidase. Octyl glucoside (25 mM)-solubilized extracts of dog pancreatic rough microsomes gave 33% conversion of [^{35}S]pre-prolactin, compared with 35% and 28% conversions when the microsomes had previously been irradiated with 1 µM- and 50 µM-ACSP; with 10 mM-octyl glucoside extracts of the membranes, the conversion was 15% (50 µM-ACSP). The peptidase activity assayed by conversion of pre-promellitin in the wheat germ system with partially purified signal peptidase from rat liver gave 33%, 37%, 29% and 37% conversions in the presence of 45 µM-, 12 µM-, 4 µM- and 0 µM of the consensus signal peptide respectively. The results showed that neither the presence of signal peptide, nor photoactivated covalent modification with ACSP, inhibited the signal peptidase. Dog pancreatic microsomal vesicles after photoactivation in the presence or absence of ACSP were treated with octyl glucoside at various concentrations, and the soluble extracts tested for their activity to convert [^{35}S]pre-prolactin to [^{35}S]prolactin post-translationally. The

Fig. 8. Photoactivation with ^{125}I -ACSP of microsomal membranes and a signal peptidase preparation from rat liver

(a) Autoradiogram displaying polypeptides modified by ^{125}I -ACSP after photoactivation with partially purified and re-constituted signal peptidase preparation from rat liver (track 1), rough microsomal membrane vesicle from rat liver (track 2), Na_2CO_3 -treated microsomal membranes from rat liver (track 3) and dog pancreatic rough microsomal membranes (track 4). (b) Shows the corresponding polypeptide profiles visualized by Coomassie Blue staining.

Fig. 9. Two-dimensional isoelectric focusing-SDS/PAGE of dog pancreatic rough microsomal membrane polypeptides

(a) Polypeptides from a total protein fraction of rough microsomal membranes detected by Coomassie Blue staining; (b) immunoidentification of the position of the docking protein by an anti-(docking protein) monoclonal antibody; (c) autoradiogram of polypeptides covalently linked to ^{125}I -ACSP after photoactivation with rough microsomal membranes not previously stripped by high salt and EDTA. Approx. 250 μg of protein samples were loaded from the acidic end of prefocused first-dimensional isoelectric gels containing Ampholines (LKB, Sweden) pH range 3.5–10. The separation in the second dimension was by SDS/PAGE [7–16% (w/v) linear gradient]. The proteins are numbered as described by Kaderbhai &

results showed that signal peptidase activity was optimally detected with about 30% conversion in the soluble extract at 25 mM concentration, irrespective of whether the membranes were irradiated in the presence or absence of the ACSP, indicating that photoactivated covalent modification of the microsomal vesicles with ACSP did not inhibit signal peptidase.

Further studies were undertaken to see if there were any structural similarities between the 45000- M_r signal peptide binding proteins and the docking protein. A total fraction of dog pancreatic microsomal membrane polypeptides was resolved by high resolution two-dimensional isoelectric focusing-SDS/PAGE as previously described by Kaderbhai & Austen (1984). A typical Coomassie Blue-stained profile is shown in Fig. 9(a). The proteins were electrotransferred onto a nitrocellulose sheet by Western blotting and probed with a mouse monoclonal antibody against the docking protein (kindly provided by Dr. Meyer) followed by immunolocalization with enzyme-linked anti-(mouse IgG). The stained immunogram (Fig. 9b) shows detection of a polypeptide of M_r 72000 in agreement with the known size of the purified docking protein, which resolves as a streak in the first dimensional isoelectric focusing but focuses as a component with a pI of about 5.0. In Fig. 9(a), the docking protein has been arrowed, close to a major protein numbered 7. Fig. 9(c) shows a comparable autoradiogram of the polypeptides from dog pancreatic rough microsomes following u.v.-irradiation with ^{125}I -ACSP, displaying the polypeptides that are modified by ACSP. The minor components labelled correspond to polypeptides numbered 5, 8 and 12 with M_r of 94000, 68000 and 57000 respectively. The polypeptide 12 has been identified as the enzyme protein disulphide isomerase (Kaderbhai & Austen, 1984). The major labelled components corresponding in size to M_r 45000 migrate as two closely similar, relatively basic, polypeptides with pI around 7.8.

DISCUSSION

The synthetic consensus signal peptide arrests the translocation activity of microsomal membrane vesicles, which hence fail to process and segregate the pre-proteins synthesized *in vitro* from mRNAs for secretory proteins (Austen *et al.*, 1984). In this study we have shown that a photoactivatable form of the consensus signal peptide, ACSP, when irradiated with light in the u.v. spectrum, also inhibits the translocation and processing activity of the microsomal vesicles.

Photoactivation of ACSP with microsomal membranes results in chemical coupling of the signal peptide to several proteins. In rough microsomal membranes the major products are components of M_r 45000, 57000 and

Austen (1984). By matching the mobility and shape of the docking protein identified in Western blot, the corresponding protein (arrowed) was traced in (a). The 45000- M_r , ^{125}I -ACSP-modified polypeptides resolved in positions corresponding to spots 21 and 22, while 70000- M_r and 59000- M_r labelled components were traced to those numbered 8 and 12. Polypeptide 12 has previously been identified as the enzyme protein disulphide isomerase (EC 5.3.4.1).

70000 (Fig. 5). Stripped microsomal membranes exhibit labelling of similar proteins, but with reduced production of M_r 57000 and 70000 adducts (compare Figs. 3 and 4 with 5 and 6). The basis for the differences in labelling between the rough and stripped microsomal membranes are not clear but could be attributed to increased number of non-selective and reactive sites exposed by salt-treatment and removal of ribosomes, and washing away many loosely attached non-specific components. Extensive binding is also observed in components of M_r below 20000 (see below).

This study clearly demonstrates that the signal sequence binds specifically and saturably to components of the endoplasmic reticulum to yield adducts of M_r 45000. As the coupling of the signal peptide is anticipated to generate an extra 2000 M_r , the native protein subunits would be slightly smaller. The polypeptides are intensely modified by the photoactivatable signal peptide in microsomal membranes derived from pancreas and liver, tissues that are active in biosynthesis and secretion of proteins; no binding is observed with components of mitochondria. The selectivity of the interaction between the signal peptide and 45000- M_r component is demonstrated by abolition of the binding by the competing presence of unmodified consensus signal peptide, and also by a natural signal peptide from ovalbumin, in a dose-dependent manner at low concentrations. The competitive effect is not exerted at equivalent concentrations by control peptides of similar sizes and hydrophobicity from parathyroid hormone or γ -endorphin. The labelling of other polypeptides, including those of M_r 70000 and 57000, is unaffected by the presence of excess signal peptides, implying that their modification may be due to non-specific binding and reactivity. Indeed, the 57000- M_r polypeptide has been identified as protein disulphide isomerase (Kaderbhai & Austen, 1984, 1985), and this enzyme may be labelled by ACSP to a significant extent due to the presence of highly reactive vicinal thiol groups in its active site (Edman, 1985). Further evidence for the non-specific nature of interaction between the ACSP and the M_r 57000 and 70000 proteins comes from the production of their adducts in the absence of u.v. light (Fig. 5). These findings therefore strongly suggest that the modification of 45000 M_r proteins results from specific, saturable binding that is a characteristic of receptor-ligand interactions, rather than non-specific interactions (Mohler *et al.*, 1980). The competition for labelling by the unlabelled consensus signal peptide in this study occurs at a similar concentration range; from 0.2 to 1 mM, over which displacement was observed by direct binding studies to rough microsomal vesicles (Austen & Ridd, 1983). The observed binding sites may be similar to the binding sites for carp pre-proinsulin in dog pancreatic rough microsomes that are not displaced by high salt concentrations, as reported by Prehn *et al.* (1980).

The possibility that the 45000 M_r -ACSP adducts are components related to or derived from SRP is unlikely because the adducts are generated in good yields after photoactivation from both the rough and stripped-microsomal membrane vesicles. The preparation of the latter membrane fraction requires high salt, which effectively depletes the subsequently isolated membrane of SRP (Walter & Blobel, 1980). Moreover, none of the subunits of SRP are of M_r 45000 (Walter & Blobel,

1982). Recently, Kurzchalia *et al.* (1986) have reported binding of nascent pre-prolactin to the 54000- M_r subunit of SRP, but this interaction requires the additional presence of polysomes attached to the nascent chain in the cytosol. Nor do the radiolabelled 45000- M_r proteins arise from covalent modification by ^{125}I -ACSP of the docking protein or its breakdown products. The properties of the 45000- M_r proteins are shown to be different from those of the docking protein with respect to their masses, pIs and binding to a monoclonal antibody against the docking protein. The two-dimensional autoradiograms show that photoactivation of ^{125}I -ACSP with microsomal vesicles does not label the docking protein, even non-specifically.

Several of the properties of the 45000- M_r signal peptide binding proteins are similar to those reported for eukaryotic signal peptidase(s). As with the signal peptidase (Lively & Walsh, 1983), the integral nature of the membrane association of the 45000- M_r signal peptide binding proteins is demonstrated by the following. (i) The adducts are only solubilized from the membrane when octyl glucoside concentration is higher than its critical micellar concentration (22 mM), a point at which the membrane phospholipids begin to form mixed micelles (Jackson *et al.*, 1982). (ii) The 45000- M_r adducts are not removed from the membrane by treating the microsomes with 0.1 M- Na_2CO_3 , a condition which converts the membrane vesicles into flattened membrane sheets and leads to over 90% (w/w) loss of loosely-associated membrane proteins (Fujiki *et al.*, 1982). From these comparisons it is tempting to speculate that the 45000- M_r ACSP-binding proteins may be signal peptidase. However, several other observations exclude this possibility. Purified reconstituted preparations of signal peptidase are not photolabelled with ^{125}I -ACSP to yield a component of 45000- M_r . Additionally, signal peptidase is a lumenally-disposed enzyme in microsomal vesicles, resistant to added proteinases, whereas in this study the 45000- M_r ACSP-binding proteins are cytoplasmically disposed. Proteinase treatment of intact functional microsomal vesicles modified with ACSP converts the 45000- M_r proteins to a radiolabelled fragment of M_r approx. 30000, which is retained in the membranes and sediments with them; the fragment is degraded further only when the membrane structure is disrupted by Triton X-100. These observations suggest that the 45000- M_r proteins have two domains, the larger 30000- M_r region being buried in the lipid bilayer or lumenally disposed, and the smaller 15000- M_r region exposed to the cytoplasmic face. Clearly, since the 30000- M_r region retains the radiolabel and resists further degradation by the endopeptidases, the ACSP binds to the larger domain of the 45000- M_r proteins. Lastly, the consensus signal peptide and ACSP inhibits the cotranslational processing activity of intact microsomal vesicles, but not their post-translational activity after detergent solubilization (results not shown). These findings therefore indicate that the abolition of the processing activity by signal peptides is not a consequence of direct inhibition of the peptidase itself but a manifestation arising from a prior event, i.e. inhibition of the translocation of the pre-proteins into microsomal vesicles *in vitro* and thus access to signal peptidase (Austen *et al.*, 1984).

The recently reported purification of signal peptidase to near homogeneity as a complex of six distinct polypeptides ranging in size from M_r 12000 to 23000 has

led to suggestions that only one of these subunits carries out the signal cleavage whereas the structural association of other subunits in stoichiometric amounts reflects their involvement in translocation of nascent polypeptide across the microsomal membrane (Evans *et al.*, 1986). In contrast to this suggestion, ACSP labels intensely in purified signal peptidase preparations and intact microsomal membranes a number of polypeptides in the M_r region of the signal peptidase subunits, but no significant protection to radiolabelling is exerted by low concentrations of the 'cold' signal peptides.

It has been suggested that signal sequence may insert into the membrane as a loop, with its basic *N*-terminus on the cytoplasmic side of the membrane, its cleavage site on the luminal side, and its core straddling the membrane in contact with phospholipid hydrocarbons (Austen, 1979). However, this transition from aqueous to apolar phase cannot account for the translocation of the following polypeptide which is predominantly hydrophilic, at least in the case of secretory proteins. Accordingly, a channel or tunnel may form in the membrane through which the nascent polypeptide chain could be threaded, the discharge being powered by polypeptide chain elongation during cotranslational translocation. The formation of protein-translocating channels would likely be a transient event, with their formation triggered by appearance of the signal sequence. In this context, it is suggested that the 45000- M_r signal-binding proteins in the microsomal membranes may act as receptors, which relay information for assembly of components for the translocation channel and/or other co- or post-translocational enzymic systems, e.g. signal peptidase, *N*-glycosylating enzymes and protein disulphide isomerase. Construction of a radioactive, photoactivatable consensus signal peptide has provided a valuable tool for the identification of these signal sequence binding proteins in the endoplasmic reticulum membrane and now paves the way for their purification and investigation of their function.

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