A Structural and Functional Analysis of the Docking Protein

CHARACTERIZATION OF ACTIVE DOMAINS BY PROTEOLYSIS AND SPECIFIC ANTIBODIES

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Docking protein is a 73-kDa integral membrane protein of the rough endoplasmic reticulum. It is essential for translocation of nascent secretory proteins into the lumen of the endoplasmic reticulum. Monoclonal and polyclonal antibodies have been generated which, in conjunction with limited proteolysis, have been used to characterize several subspecies of docking protein. These proteolytic fragments have been analyzed with respect to the various functions ascribed to docking protein which can be assayed in vitro.

Proteolytic digestion of membrane-associated or of affinity-purified intact docking protein showed that elastase cleavage generates a 59-kDa soluble fragment and one of 14 kDa which contains the membrane anchoring domain; trypsin as well as endogenous proteolysis generates a 46-kDa fragment, leaving a 27-kDa domain containing the membrane anchor. This 27-kDa fragment can be reduced to a 13- and a 14-kDa piece by elastase digestion.

The characteristics of these various subspecies were examined. The 59-kDa soluble fragment, which can reconstitute full translocation activity to docking protein-depleted microsomes (Meyer, D. I., and Dobberstein, B. (1980) J. Cell Biol. 87, 503–508) was capable of releasing a signal recognition particle-mediated translation arrest. The 46-kDa fragment was neither able to reassociate with nor to reconstitute the activity of docking protein-depleted microsomes. Moreover, this fragment was unable to release a signal recognition particle-mediated arrest. This suggests that the 13-kDa fragment (the difference between 46 and 59 kDa) is both essential for association with the membrane, and for the release of translation arrests.

Passage through the membrane of the rough ER represents the step in which secretory proteins leave their site of synthesis, the cytosol, and enter the pathway leading to the cell surface. Those proteins destined to leave the cytosol are selected by virtue of a specific recognition mechanism which has been elucidated in molecular terms over the past decade (for review, see Refs. 1–3). The signal sequence of secretory proteins is recognized by SRP shortly after its synthesis and usually before completion of the nascent chain (4). This event is followed by a docking maneuver in which the SRP, still bound to the nascent chain, interacts with a 73-kDa ER-specific receptor, the docking protein (5). At this point, by some as yet undefined mechanism, the nascent peptide is translocated across the membrane of the rough ER.

In a wheat germ cell-free system certain aspects of this SRP:protein interaction have been characterized. Translations of mRNA encoding secretory proteins are arrested at a chain length of 60–70 amino acids by the addition of SRP at the onset of translation (5, 8). This arrest can only be released by the addition of docking protein or docking protein-containing membranes (5–8). These results have been interpreted to imply the necessity of a translation arrest as a feature of efficient targeting of secretory proteins to the rough ER (1, 4, 8). In this way completion of nascent secretory proteins can only be achieved in close proximity to the membrane across which the protein must be transferred, i.e. the rough ER.

Thus docking protein represents the cross-roads of cytoplasmically and membrane-localized events in the secretory pathway. Active docking protein was originally isolated as a proteolytically derived, water-soluble cytoplasmic domain (M, = 60 kDa) which had the unique property of being able to recombine with docking protein-free ER membranes and reconstitute translocation activity in vitro (9, 10). This reconstitution was dependent upon the restoration of an electrostatic interaction between the 60-kDa docking protein fragment and the membrane.

All the features of docking protein pointed out above show it to be a multifunctional component of the rough ER. First and foremost, docking protein is the receptor for SRP and has a specific affinity for it. Second, docking protein is responsible for releasing the SRP-induced arrest of translation observed in the wheat germ system. Last, the 60-kDa docking protein fragment can recombine with some, as yet unidentified, component in the ER membrane in a reconstituted system. To better understand the molecular details of the individual reactions in which docking protein participates, it is necessary to fractionate the molecule into specific functional domains. By making use of the various in vitro assays which have been developed, each isolated domain can be tested, and ultimately function can be related to a defined structural feature of docking protein.

We report here that several subspecies of docking proteins have been proteolytically generated which were identified by...
different anti-docking protein antibodies. Using various in vitro assays it was possible to ascribe specific functions to particular domains of these docking protein fragments, including the ability to reconstitute proteolyzed microsomes, the effectiveness in releasing an SRP-induced translation arrest, and the interaction with and anchoring in the ER membrane.

**EXPERIMENTAL PROCEDURES**

Dog pancreas was generously provided by Sandoz AG, Basel, Switzerland. mRNA was prepared from MOPC-41 cells as described (11, 12). [35S]Methionine was purchased from New England Nuclear and rabbit reticulocyte lysate system from Staehelin (Basel, Switzerland). Wheat germ lysate was prepared as described by Grossman et al. (13). Protease and protease inhibitors were obtained from the following companies: trypsin and phenylmethylsulfonyl fluoride from Sigma, elastase from Merck & Co., Darmstadt, West Germany, and trasylol from Boehringer Mannheim. Phosphate-buffered saline without calcium and magnesium was purchased from Biochrom KG (Berlin, Germany).

Preparation and Treatment of Microsomal Membranes—Rough microsomes from dog pancreas were prepared as described previously (11, 12). Microsomal membranes were carbamylated washed according to the method of Fujiki et al. (14) with the following modifications: rough microsomes (A260 = 60/μl) were diluted 1:10 with 0.1 M Na2CO3, pH 11, and centrifuged through a sucrose cushion in 0.1 M Na2CO3 for 90 min at 105,000 × g. The pellet was resuspended in 0.1 M Na2CO3 and again centrifuged through a cushion containing 50 mM triethanolamine, pH 7.5, and 0.25 M sucrose. Protease treatment of microsomes was carried out for 1 h at 0°C. The reactions were terminated by the addition of phenylmethylsulfonyl fluoride to a concentration of 200 μg/ml. Proteolyzed extracts and insoluble pellets were separated as described by Meyer and Dobberstein (9).

Affinity Purification of Docking Protein—Rough microsomes were suspended in solubilization buffer containing 20 mM HEPES, pH 7.5, 200 mM KOAc, 5 mM MgCl2, 1 mM DTT, and 40 μg/ml phenylmethylsulfonyl fluoride to a concentration of 50 A260/ml. The nonionic detergent Nikkol (octaethylene glycol n-dodecyl ether, Nikko Chemicals, Tokyo, Japan) was added to a final concentration of 1% (w/v). Insoluble material was removed by centrifugation for 1 h at 105,000 × g. The supernatant was loaded onto a column which was packed with carboxymethyl (CM)-Sephadex (Pharmacia, Uppsala, Sweden). The column was washed extensively with solubilization buffer. Bound material was then eluted with the same buffer without DTT containing 350 mM KOAc. This fraction was loaded onto an affinity matrix to which monoclonal anti-docking protein antibodies were bound. The affinity matrix was prepared using Affi-Gel 10 (Bio-Rad) as described by Meyer et al. (15). The affinity column was eluted with 0.2 M acetic acid containing 0.5% Nikkol. The eluted material was immediately neutralized with 1 M Tris base and subsequently dialyzed against solubilization buffer containing 0.5% Nikkol.

Preparation of Rabbit Anti-docking Protein Antibodies—The polyclonal antisera against the 59-kDa elastase-generated docking protein fragment has been described previously (16). Antiserum against the 27-kDa membrane inserted fragment was obtained by immunization via the popliteal lymph node (17). The antigen was isolated from solubilized dog pancreas microsomes by affinity chromatography on monoclonal antibody columns. The 27-kDa fragment was separated from the intact 73-kDa docking protein molecule, also obtained by this procedure, by preparative SDS-polyacrylamide gel electrophoresis.

**Polyacrylamide Gel Electrophoresis—**Proteins were separated and analyzed by SDS-polyacrylamide gel electrophoresis on 10–15% gradient gels unless otherwise stated. Protein profiles were visualized using the silver stain method of Ansgore (18). Polyacrylamide gels were fixed for fluorography in 10% trichloroacetic acid and treated with ENHANCE (New England Nuclear) for 1 h. Gels were washed, dried, and placed in contact with Kodak X-Omat AR film at −80°C. Apparent molecular weights were determined using a mixture of the following proteins as reference standards: rabbit muscle myosin (205 kDa), rabbit muscle phosphorylase b (92.5 kDa), bovine serum albumin (68 kDa), chicken ovalbumin (45 kDa), bovine pancreatic α-chymotrypsinogen (25 kDa), and horse heart cytochrome c (12.5 kDa).

**Immunoblotting of Microsomal Proteins—**Immunoblots were performed using a modification of the procedure developed by Burnett (19). Proteins were electrophoretically transferred to nitrocellulose filters (0.2 μm, Schleicher and Schuell). Transfer was verified by protein staining using 0.2% Ponceau S solution in 3% trichloroacetic acid (Serva, Heidelberg, West Germany). The bound dye was completely removed from the proteins by incubation for 30 min in blotting buffer (phosphate-buffered saline, 10% newborn calf serum). Subsequently the filters were incubated for 1 h in a solution of the first antibody which was diluted to an appropriate concentration in blotting buffer. Rabbit antisera were usually diluted 1:500 and mouse ascites fluid 1:1000. Biots were then washed three times for 5 min with blotting buffer. Trion X-100 (0.05%) was included in the second washing step. This was followed by a 1-h incubation in the presence of the second antibody peroxidase-labeled which was diluted in blotting buffer. For rabbit antibodies a 1:1000 dilution of goat anti-rabbit IgG-peroxidase conjugate (Medac, Hamburg, West Germany) was used, and for mouse antibodies a 1:300 dilution of peroxidase-labeled sheep anti-mouse IgG antibodies (Sigma). The filters were washed three times for 15 min with phosphate-buffered saline. The second wash contained 0.05% Trion X-100. The immunoblots were briefly equilibrated with 50 mM Tris/HCl, pH 7.5, and developed with 0.5 mg/ml diaminobenzidine (Sigma) in 50 mM Tris/HCl, pH 7.5, containing 0.03% H2O2. In Vitro Assays—Cell-free translations in reticulocyte lysate were carried out as described by Dobberstein et al. (21). The reconstitution of proteolyzed rough microsomes (RM) using the elastase-generated fragment of docking protein (docking protein fragment) was performed according to Meyer and Dobberstein (9). The rebinding of the docking protein fragment to RM, was characterized using the membrane affinity factor isolation assay published by Meyer and Dobberstein (22) in conjunction with the immunoblot procedure described above. Translations in the wheat germ cell-free system and assays for release of the SRP-induced translation arrest were carried out according to Gilmore et al. (6). SRP was prepared as described by Walter et al. (23). Units of SRP are those which have been defined previously (23).

**RESULTS**

In order to facilitate the interpretation of the data presented below, the deduced domain model of the docking protein is shown in Fig. 1. The comparison of docking protein fragments which were created by the limited proteolysis of microsomal membranes and recognized by polyclonal or monoclonal antibodies enabled the construction of this structural model.

**Docking Protein Is an Integral Membrane Protein—**Several authors reported that intact docking protein can be solubilized from dog pancreas microsomes only by the use of detergents (7, 16). This suggests, but does not prove, that docking protein

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1 Portions of this paper (including part of “Experimental Procedures,” Table I, and Fig. 11) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9600 Rockville Pike, Bethesda, MD 20814. Request Document No. 85M-216, cite the authors, and include a check or money order for $1.60 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

![Fig. 1. Domain structure of the docking protein as determined by limited proteolysis and by mapping of monoclonic determinants. Numbers indicate the apparent molecular weights (in kilodaltons) of the protein domains as determined by polyacrylamide gel electrophoresis. Pab and Mab refer to approximate recognition sites for polyclonal and monoclonal anti-docking protein antibodies, respectively.](image-url)
is embedded in the lipid bilayer. The carbonate wash method of Fujiki et al. (14) would demonstrate in a more stringent manner that docking protein is indeed anchored in the microsomal membrane. Samples of rough microsomes were thoroughly washed at pH 11 and soluble and insoluble material was analyzed on immunoblots. It is clear from the results presented in Fig. 2 that docking protein was retained in the membrane following exposure to 0.1 M carbonate at pH 11 (compare lanes 1 and 3), whereas no docking protein could be detected in the bulk of the microsomal proteins comprised largely of secretory and peripheral membrane proteins (lane 2). This indicates that at least part of the docking protein is embedded in the lipid bilayer and that it represents a genuine integral membrane protein. Of the four different anti-docking protein monoclonals (see Miniprint), all recognized a pH 11 insoluble 27-kDa species as well, which thus represent a membrane-anchored degradation product of docking protein (refer to Fig. 1). Neither the 27-kDa fragment nor the intact 73-kDa docking protein molecule were released from the microsomal membrane by treatment with 500 mM KI or 2 M NaCl. Furthermore, both species were cloud point-precipitable by the nonionic detergent Triton X-114 (24), further proof for their insertion in the lipid bilayer (data not shown).

The notion that the 27-kDa protein fragment is derived from docking protein by endogeneous proteolysis is supported by the following facts: (a) docking protein is very susceptible to proteases (9). Dog pancreas microsomes contain a high level of protease precursors including trypsinogen, chymotrypsinogen, procarboxypeptidases, and elastase precursor (25, 26). The amount of the 27-kDa fragment varies between different microsomal preparations and is inversely proportional to the amount of intact docking protein found (compare Fig. 2, lane 1, with Fig. 3, lane 2). (b) The 27-kDa fragment is recognized by four clearly different monoclonal antibodies (see Table I in the Miniprint Section). (c) A polyclonal rabbit antiserum raised specifically against the affinity-purified 27-kDa fragment recognized the 73-kDa intact docking protein on immunoblots (Fig. 3, lane 2).

**Protease Digestion of Docking Protein**—Meyer and Dobberstein (10, 16) reported that elastase cleaved a 60-kDa fragment from the intact 73-kDa docking protein which can be detached from the membrane by increasing salt concentrations. Such treatment allowed a more precise mapping of the epitopes against which the different monoclonal antibodies are directed. As expected, the 73-kDa docking protein (Fig. 4, lane 1) could no longer be detected after treatment with 3 μg/ml elastase and 500 mM KCl (lane 2). In the supernatant fraction two new fragments, with apparent molecular masses of 59 kDa (a more exact calculation of the molecular mass of the fragment previously referred to as 60 kDa) and 13 kDa, were

![FIG. 2. Docking protein is a 73-kDa integral microsomal membrane protein.](image)

1 2 3

92,500
68,000
45,000
25,000
12,500

![Fig. 3. A polyclonal antiserum raised against the 27-kDa fragment recognizes the intact 73-kDa docking protein molecule. Lane 1 shows the affinity purified 27-kDa fragment preparation which was used as immunogen in rabbits visualized by silver staining. Lanes 2–6 represent immunoblots incubated with anti-27-kDa docking protein fragment antibodies, and lane 7 is an immunoblot reacted with anti-59-kDa docking protein fragment antibodies. Lane 2, salt-washed rough microsomes; lane 3, microsomal membrane incubated at 0 °C for 1 h with 3 μg/ml trypsin and washed with 0.5 M KCl; lane 4, elastase/high salt supernatant of the microsomal preparation shown in lane 3; lane 5, rough microsomes incubated at 0 °C for 1 h with 5 μg/ml trypsin and washed with 0.5 M KCl; lanes 6 and 7, trypsin/high salt supernatant derived from the microsomal membranes described in lane 5.](image)
detected by the monoclonal antibodies (lane 3). The 59-kDa fragment is derived from the 73-kDa intact docking protein, as was previously reported (16). Since it is readily washed off of the membrane by high salt concentrations without detergent it does not contain the membrane inserted portion of the intact molecule. The 13-kDa species represents one of the cleavage products of the 27-kDa docking protein fragment which could be detected by monoclonal and polyclonal antibodies. The other product was a fragment containing the membrane anchor of 14 kDa (see below).

Several studies have shown that trypsin can replace elastase in generating extracts of rough microsomes capable of functionally reconstituting RM (6, 9, 27). It was never determined whether the fragment of docking protein generated by trypsin was identical to the elastase-derived docking protein fragment. As can be seen in Fig. 5, increasing amounts of trypsin generated a series of fragments between 60 and 45 kDa which were detected by anti-docking protein antibodies. At the trypsin concentrations which were previously shown to yield active extracts (9), a 59- and a 56-kDa fragment were present in small amounts (panel A). At higher concentrations, where no activity was seen, the predominant species had an apparent molecular mass of 46 kDa (panel B). The analysis of the functional state of this fragment is presented later in this report.

At still higher trypsin concentrations the 46-kDa fragment was degraded. Since the 46-kDa species does not contain the membrane inserted domain and is not stained by any monoclonal antibody (panel B), it must be part of the 59-kDa elastase-generated fragment excluding the epitopes recognized by the monoclonals (see Fig. 1). This places the 46-kDa domain at the end of the docking protein molecule most distal from the membrane. The data shown in Fig. 3 support this location of the 46-kDa domain. The polyclonal antiserum raised against the 27-kDa membrane-inserted docking protein fragment also recognized the 59-kDa elastase-generated fragment (lane 4) but failed to stain the 46 kDa trypsin generated fragment (lane 6). Since in this experiment the starting material did not contain a high amount of the 27-kDa species, the aforementioned 13-kDa fragment is not detectable in the elastase extract as is shown in Fig. 4.

**Affinity Purification of Docking Protein**—All experiments described so far were performed on intact microsomal membranes of which docking protein constitutes only a minor component (according to our estimation of silver-stained SDS-polyacrylamide gels, less than 0.1% of the total microsomal protein). For a more detailed structural and functional analysis of the molecule it was advantageous to have homogeneous preparations. The published procedure for the isolation of the pure 73-kDa docking protein is very cumbersome and yields only small amounts of material (7). Antibodies from different hybridoma cell lines were therefore used to develop a simple, rapid affinity-based procedure for the large-scale purification of the intact docking protein.

Monoclonal antibodies purified from ascites fluid were covalently coupled to a solid support (see "Experimental Procedures"). This affinity matrix was used to purify docking protein from solubilized pancreatic rough microsomes in a two-step procedure. Solubilized docking protein was first enriched by ion exchange chromatography as described by Meyer and Dobberstein (10). The fraction eluted with 350 mM KOAc (Fig. 6, lane 1) was applied to the affinity column. Bound material was eluted with 0.2 M acetic acid and immediately neutralized with 1 M Tris base. 73-kDa docking protein was purified selectively and quantitatively together with the 27-kDa fragment (Fig. 6, lane 3). Material purified using this procedure was active as evidenced by its ability to release the SRP-induced translation arrest in a wheat germ cell-free translation system (data not shown).

Use of affinity-purified material enabled us to visualize the membrane-associated docking protein domain (which is not recognized by any of the antibodies) directly on silver-stained SDS-polyacrylamide gels. Limited proteolysis of pure 73-kDa docking protein with 1 μg/ml elastase generated the familiar 59-kDa fragment (Fig. 7, lanes 2 and 5). An additional product of elastase digestion was a species with a molecular weight of 14 kDa seen only on silver-stained polyacrylamide gels (lane 2). As shown in Fig. 7, lane 5, this polypeptide fragment was not stained by the monoclonal antibodies on immunoblots. We therefore conclude that this 14-kDa docking protein fragment contains the membrane-associated domain of docking protein which remains when a 59-kDa and a 13-kDa fragment is elastolytically removed from the 73- and 27-kDa docking protein species, respectively. Note the faint appearance of the 13-kDa fragment on the immunoblot in lane 5 (compare also to Fig. 4, lane 3). Somewhat higher elastase concentrations were necessary to split one molecule of 27 kDa into one molecule each of 13 kDa (recognized by polyclonal and monoclonal antibodies) and 14 kDa, the latter representing the...
membrane inserted protein portion.

The results obtained with proteolysis can be summarized as follows (refer to Fig. 1). Docking protein is a 73-kDa integral membrane protein. Elastase will remove a 59-kDa domain leaving a 14-kDa piece which contains the membrane anchor. Trypsin, at very low concentrations, will also generate a fragment of about 60 kDa, but the major product of trypsination is 46 kDa. This 46-kDa piece is fully contained in the 59-kDa elastase fragment and represents the part of docking protein most distal to the membrane. A naturally occurring degradation product is the membrane-associated 27-kDa fragment. Cleavage of the 27 kDa by elastase yields fragments of 13 kDa, which is recognized by the antibodies, and 14 kDa, the membrane anchor, which is not.

Functional Analysis of the Docking Protein Domains—Several functions and biological activities of docking proteins have been described in the literature. The first is the absolute requirement for docking protein in order to get a nascent secretory protein translocated across the microsomal membrane (5, 9). Microsomal membranes which have been treated with low amounts of proteases (RM) are inactive in protein translocation. However, after the addition of purified 59-kDa docking protein fragment they regain their full activity when included in an in vitro translation system (9, 10). Additionally, docking protein has been identified as the molecule which binds to SRP and is responsible for the release of the SRP-induced translation arrest in the wheat germ cell-free system (5, 7). We therefore tried to correlate the structural domains of docking protein which we have prepared with the different activities in various in vitro assay systems.

The ability of docking protein fragments to restore translocation activity to proteolyzed microsomes is shown in Fig. 8. The 59-kDa elastase-generated fragment was able to restore the activity of protease inactivated microsomal membranes (in a rabbit reticulocyte lysate translation of immunoglobulin light chain mRNA) (Fig. 8, lanes 3 and 4) (9, 10). This is indicated by the appearance of the authentic lower molecular weight form of the immunoglobulin light chain and disappearance of the precursor form (lane 4). This processing activity is strictly coupled to co-translational transfer of the nascent peptide chain into the microsomal lumen (11, 12). In contrast, the trypsin extract, consisting primarily of 46-kDa docking protein fragment, was ineffective in reconstituting the translocation ability of inactivated microsomes (Fig. 8,
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FIG. 6. Affinity purification of docking proteins on a monoclonal antibody affinity matrix. Dog pancreas microsomal proteins were solubilized (see "Experimental Procedures") and were enriched for the docking protein on a CM-Sephadex ion exchange column. The material eluted from CM-Sephadex at 350 mM KCl (lane 1) was applied to an affinity matrix (monoclonal antibody 12E3 coupled to Affi-Gel 10). The proteins which were not bound by the antibody are shown in lane 2. Proteins which could be eluted with 0.2 M acetic acid, 0.5% Nikkol are visualized in lane 3. Proteins were stained by silver as described.

It was therefore of interest to determine if the inability of this fragment to reconstitute activity could be accounted for by its failure to rebind to proteolyzed microsomes. The 59-kDa docking protein fragment rebound almost quantitatively under low salt concentrations to protease-treated microsomes (Fig. 9, lanes 2 and 5). Microsomes which could be centrifuged out of the incubation mixture contained most of the exogenously added elastase-generated docking protein fragment. In contrast, no rebinding of the 46-kDa trypsin-generated fragment was observed under the same conditions. Instead, the added fragment was found in the supernatant (Fig. 9, lanes 3 and 6). Aggregation of docking protein fragment was not observed in either extract as determined by centrifugation in the absence of membranes (data not shown). Thus the portion of the 59-kDa docking protein fragment involved in rebinding must lie in the 13-kDa piece not present in the 46-kDa fragment. Note that this is the same piece which can be cleaved from the 27-kDa membrane-bound fragment.

Since solubilized 73-kDa docking protein is the receptor for SRP in the rough ER, we investigated the possibility that this activity is preserved in the different docking protein fragments. The surest assay for "SRP receptor activity" is the ability of docking protein to release the SRP-induced arrest of translation which is observed in wheat germ cell-free translations (5, 6, 8). Shown in Fig. 10 is the typical arrest of
translation brought about by SRP (lanes 1 and 5 compared to lanes 2 and 6). This arrest was relieved by the addition of 73-kDa docking protein as shown in lanes 3 and 7. As would be expected from the reconstitution data already presented, the 59-kDa elastase fragment also released the block (lane 8) whereas the 46-kDa tryptic fragment did not (lane 4). The immunoblot, shown in lanes 9 and 10, indicates that the 59-kDa elastase fragment preparation shown to be capable of releasing the arrest was completely free of intact 73-kDa docking protein.

**DISCUSSION**

The structural model depicted in Fig. 1 represents a useful tool with which to interpret the available functional and immunological data on docking protein. The epitopes of all four different monoclonal antibodies which reacted with docking protein on immunoblots are located on a 13-kDa domain within the complete molecule. This domain is possibly responsible for the basic character of docking protein, which was reported by Meyer and Dobberstein (10). We found that only those docking protein fragments including this domain bound to the cation ion exchanger CM-Sephadex at low salt concentrations, e.g. the 59-kDa and the 27-kDa fragment (data not shown). The two polyclonal rabbit antisera recognized at least two main antigenic regions located on the 13-kDa domain close to the membrane inserted portion of the molecule, and on the outer 46-kDa domain.

The findings presented here are particularly interesting with respect to the ability of tryptic digestion to generate active docking protein fragments. Using very low concentrations (up to 1.0 μg/ml) fragments were obtained whose activity seemed to be contingent upon the presence of small amounts of the 59-kDa species. At higher concentrations (2–20 μg/ml) only 46-kDa fragments could be detected. Such fragments were inactivate in both reconstitution and block release assays. It is likely, therefore, that previously reported successes...
in the ability of trypsin to generate extracts active in reconstitution were due to the presence of the larger 59-kDa fragment. This fact was reflected in the actual degree of reconstitution. Extracts generated by trypsinization were able to effect levels of reconstitution approaching 30-40%/eq (9, 27), whereas elastase extracts, which consisted almost exclusively of 59-kDa docking protein fragments were able to reconstitute levels in excess of 85%/eq (10).

It is still unclear through which interaction(s) the fragments were able to rebind to the microsomal membrane and to reactivates through the receptor interactions. The entity which acts as the "docking protein fragment" is not yet identified. It might be a residual or membrane-associated piece of docking protein, e.g. the 14-kDa fragment, a yet unidentified microsomal protein or simply the phospholipids of the microsomal membrane. Preliminary experiments indicate that the rebinding reaction is abolished by treating the microsomes with high trypsin concentrations. This would indicate that the "receptor" is a protein. Only docking protein fragments including the 13-kDa domain were able to rebind to proteolyzed membranes. The basic character of this docking protein domain could be important since the interaction is sensitive to high salt (9).

The use of the monoclonal antibodies to inhibit the reconstitution of RM, with the 59-kDa docking protein fragment, or to block translocation of nascent peptides into intact microsomes in a wheat germ cell-free system, has not been successful. This suggests that the different epitopes recognized by the monoclonal antibodies are probably not specifically involved in re-binding the elastase fragment or the interaction of docking protein with SRP.

The ability of various docking protein-derived domains to release SRP-induced translation arrest paralleled their activity in reconstitution studies. The 59-kDa fragment, but not the 46-kDa fragment, was active in block release. Some controversy exists in the literature as to whether 59-kDa docking protein fragment is capable of bringing about block release (5, 6). We have recently tested several preparations of 59-kDa docking protein fragments. Some were active in block release, whereas others were not. A common feature of all of the preparations which were incapable of releasing the block was that the 59-kDa docking protein fragment had become aggregated to such an extent that it could be almost quantitatively sedimented by centrifugation at 10,000 × g for 30 min. In those preparations which were active, little if any 59-kDa docking protein fragment could be found in such pellets.6

An alternative explanation for the ability of 59-kDa docking protein fragment to release the block proposed that contaminating 73-kDa docking protein was present in sufficient amounts to bring about release (6) (as can be seen, for example, in the extract shown in Fig. 9, lane 2). Examination of the immunoblot in Fig. 10 shows the absence of 73-kDa docking protein in the preparation of 59-kDa docking protein fragment used in our block release experiments. Based on a study in which the minimum amount of 73 kDa needed for release in a 25-μl translation was determined to be 8 ng, it was found that as little as one-tenth of this amount of 73 kDa docking protein, or 0.8 ng, was still visible on immunoblots (data not shown). Thus, if contaminating 73-kDa docking protein was present in this particular 59-kDa docking protein fragment preparation fragment responsible for the observed release, it would certainly have been visible on immunoblots, which, as is clear from Fig. 10, it was not.

Coupling in vitro assays for docking protein function with specific antibodies and proteolysis has enabled the assignment of specific functions to precise regions within the molecule. Moreover, the various fragments which can now be obtained by proteolysis of large amounts of affinity-purified docking protein can be used as antigens in the production of other specific antibodies. It is hoped that in this way, further structural and functional attributes of the translocation machinery of rough ER can be determined.

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SUPPLEMENTAL MATERIAL TO

A STRUCTURAL AND FUNCTIONAL ANALYSIS OF THE DOCKING PROTEIN: CHARACTERIZATION OF ACTIVE DOMAINS BY PROTEOLYSIS AND SPECIFIC ANTIBODIES

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GENERATION OF MONOCLONAL ANTIBODIES AGAINST THE DOCKING PROTEIN FROM CANINE PANCREAS

Preparation of the antigen:

Intact 73 kDa DP was isolated from salt washed dog pancreas microsomes which were prepared as described by Blobel and Dobberstein (11,12). Rough microsomes were extensively washed with 0.1 M sodium carbonate (pH 11) using the method of Fujiki et al. (16). Membranes were solubilized by the addition of the nonionic detergent N,N,N,N-tetraethylenglycol dodecyl ether, Nikko Chemicals Co., Tokyo, Japan) to a final concentration of 1% and neutralized by dialysis against 180 mM KCl, 20 mM HEPES, pH 7.5, 0.5% Nikkol, 1 mM DTT, and 40 pg/ml PMSF.

Solubilized material was loaded onto a column of cyanogen bromide (CN)-Sephadex (Pharmacia, Uppsala, Sweden) equilibrated with 200 mM KCl, 20 mM HEPES, pH 7.5, 1 mM DTT. DF-containing material was eluted with 350 mM KCl and further separated by preparative 5-15% SDS polyacrylamide gel electrophoresis. Protein bands were visualized by immersing the gel in 400 mM KAc for 10 min. The molecular weight region containing the DP was cut out with a razor blade and the gel pieces were electroeluted overnight (16). Eluted proteins were dialyzed against 250 mM NaCl. 20 mM HEPES, pH 7.5, 1 mM DTT and 10 pg/ml PMSF. The last dialysis buffer contained 50% glycerol in order to concentrate the material. This preparation was analyzed by SDS polyacrylamide gel electrophoresis and immunoblotted with polyclonal antibodies against DP (Fig. 11). The preparation of this polyclonal antiserum against the 59 kDa elastase was described previously (16).

Immunization procedure and cell fusion:

DP, prepared in this way was used to immunize 8 week old male BALB/c mice. Mice were first injected with approximately 20 pg protein in complete Freund's adjuvant followed by two injections with 5 pg protein in incomplete Freund's adjuvant in two week intervals. The antibody titers of the mouse sera were determined using the dot test described below and by immunoblots of dog pancreas microsomal proteins. After 4 weeks one selected mouse received twice (on day 3 and 7) before cell fusion) 5 pg protein in PBS i.p. Spleen cells of this mouse and Sp2-0 myeloma cells (provided by Dr. H. Schell, EMBL Heidelberg) were fused using polyethylene glycol 4000 (Both, Karlsruhe, West Germany). Cells were distributed into six 20 well Costar plates containing 30,000 chicken macrophages per well (chicken macrophages were generously provided by Drs. J. Bahn and H. Beug, EMBL Heidelberg). Hybrid cells were selected in RPMI medium containing 10% fetal calf serum and the HAT components. 2 to 3 weeks after fusion, 117 wells containing growing clones were screened for the production of antibodies directed against dog pancreas microsomes.

Screening assay:

Approximately 1 ml of salt-washed (60 A280 units/ml) or pH 11 treated (7 A280 units/ml) dog pancreas microsomes was spotted on a nitrocellulose filter (0.2 μm pore size, Schleicher and Schuell, Dassel, West Germany) which was subsequently saturated for 30 min with PBS containing 10% newborn calf serum. The filters were then incubated for 1 hour at room temperature with cell culture supernatant, washed three times for 5 min with saturation medium and finally incubated for 1 hour with a 1:300 dilution of a sheep anti-mouse immunoglobulin-peroxidase conjugate (Sigma Chemical Co., St. Louis, U.S.A.) in PBS containing 10% newborn calf serum. After three washes for 5 min with PBS, bound antibodies were visualized with diaminobenzidine (0.5 mg/ml, Sigma Chemical Co., St. Louis, U.S.A.) and H2O2 (0.03%) in 50 mM Tris/HCl buffer, pH 7.5 (20).

Characterization of monoclonal antibodies

Six different wells gave a positive reaction in this screening assay. Positive hybrid cells were cloned by limiting dilution on chicken macrophages. Ascites fluid was produced in pristane-treated BALB/c mice. Antibody subclasses were typed using subclass specific rabbit antisera (Litton Bionetics Inc., Kensington, U.S.A.). The characteristics of five stable hybrid cell lines secreting monoclonal antibodies against canine DP are listed in Table 1.

Antibodies from all five stable positive hybridoma clones were tested for their ability to recognize the DP in immunoblots of dog pancreas microsomal proteins. Four out of five monoclonals detected the DP In these preparations as a band with an apparent molecular weight of 73 kDa.

Table 1 Characteristics of different monoclonal antibodies detected against the DP of canine pancreatic rough microsomes

| Clone | IgG2 | IgE1 | IgE2 | IgE3 | IgE4 |
|-------|------|------|------|------|------|
| Reactivity with the 73 KDa canine DP, the 59 KDa, and the 27 KDa fragments on immunoblots | + | + | + | + | + |
| Cross-reactivity with mouse, rat and human DP on immunoblots | + | + | + | + | + |
| Affinity purification of DP by antibody coupled to Affigel 10 | n.d. | + | + | + | + |
| Immunofluorescence analysis showing ER staining pattern in MCDC cells | + | + | + | + | + |

n.d. = not determined