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THE HUMAN DOCKING PROTEIN DOES NOT ASSOCIATE WITH THE MEMBRANE OF THE ROUGH ENDOPLASMIC RETICULUM VIA A SIGNAL OR INSERTION SEQUENCE-MEDIATED MECHANISM

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Docking protein (DP, or SRP receptor) is an essential component of the cellular machinery that mediates the targeting of nascent secretory and membrane proteins to the rough endoplasmic reticulum (ER). In this study we have investigated the nature of its own targeting to its site of function, the rough ER. Using an *in vitro* transcription-translation system we demonstrate that DP is not inserted into the membrane via a classical SRP/DP-mediated process (in contrast to human ribophorins), nor via hydrophobic insertion sequences (in contrast to cytochrome b_5). Instead, we suggest that membrane assembly of DP is receptor-mediated; requiring the presence in the membrane of other proteins that mediate its targeting and insertion.

Since the discovery and characterization of the components that mediate the signal sequence-dependent targeting of secretory proteins to the rough endoplasmic reticulum (ER), SRP and docking protein (DP) (1,2,3), the question of how the DP itself is targeted has frequently arisen. Functionally, it represents the site to which nascent polypeptides (that have SRP attached to their signal sequence) are targeted as the first step in their entry into the secretory pathway (4,5). It is therefore of considerable interest to determine whether this essential component of the targeting apparatus, that itself has to be targeted to the very same membrane, makes use of the system to which it belongs, or if another mechanism is operating.

The primary sequence of canine and of human DP (6,7) shows that there is no classical cleavable signal sequence, as the N-terminus of DP has the identical amino acid sequence compared to the one deduced from the cDNA. This does not, however, rule out the possibility that this molecule contains internal signals that are capable of triggering the recognition machinery (8). Lauffer *et al.* (6) reported that there are two stretches of hydrophobic amino acids, near to the N-terminus, that could be involved in membrane insertion or anchoring. Both of these sequences are, however, interrupted by positive charges that would render them suboptimal as targeting and/or insertion signals.

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Abbreviations: DP, docking protein; ER, endoplasmic reticulum; RI ribophorin I; RII ribophorin II; RM_{KN}, salt-washed, nuclease-treated rough microsomes; SRP, signal recognition particle.

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Recently, Tajima *et al.* (9) have shown that DP can be isolated as a complex containing a 30 kDa integral membrane protein. They speculate that this smaller molecule, referred to as the ß-subunit of the DP, may assist in anchoring the DP itself (referred to as the α-subunit) to the membrane of the rough ER. This would provide an attractive mechanism for the SRP/DP-independent targeting and association of the DP itself with the ER membrane. In order to investigate these questions in detail, we have used a full size cDNA coding for human DP to study its assembly into canine pancreatic microsomes *in vitro*. Using the appropriate controls for SRP/DP-dependent and independent ER membrane proteins, we report here that DP does not assemble into the ER membrane via either a classical signal or an insertion sequence (10). Instead, DP can become stably assembled into microsomes, even post-translationally, when an excess of membranes are present. We interpret these results in light of the suggestion that the DP may require association with another integral membrane protein for its correct targeting and assembly.

MATERIALS AND METHODS

In vitro transcription and translation. Full size cDNA clones for human DP (7) and human ribophorins I and II (11) were subcloned into the EcoRI site of pGEM2. The artifical rat cytochrome b_5 clone (12) was subcloned into a PstI and EcoRI restricted pGEM1 vector. Capped mRNAs were transcribed *in vitro* as described (13). 2 µl of the *in vitro* transcripts were translated in a wheat germ cell-free system (14) in a final volume of 25 µl. All wheat germ translations were incubated for 60 min at 25° C. Where indicated SRP (40 U/ml) and/or dog pancreas microsomes (RM_{KN}) (~0.8 A_{280}/ml) were included. SRP was isolated from dog pancreas rough microsomes according to Walter and Blobel (1). Nuclease-treated dog pancreas microsomes were prepared as described by Blobel and Dobberstein (15). SDS-PAGE of cell-free translation products on 10-15% polyacrylamide gradient gels and subsequent processing for fluorography were essentially as described (16).

Treatment of microsomes: Salt-washed, nuclease-treated rough microsomes (RM_{KN}) were carbonate extracted accoding to the method of Fujiki *et al.* (17) with the modifications described by Hortsch *et al.* (18). Membrane association after *in vitro* translation was monitored as following. 10 µl of the wheat germ translation mixture were diluted with 90 µl 0.1 M Na_2CO_3 (pH 11) and kept for 15 min on ice. Membranes were recovered by centrifugation over a 50 µl 0.25 mM sucrose cushion (pH 11) in a Beckman airfuge for 15 min at 18 psi and directly dissolved in SDS-PAGE sample buffer. The supernatant fractions were neutralized with 1 M HCl and precipitated for 15 min on ice with a final concentration of 25% aceton. Precipitated proteins were pelleted by centrifugation in an Eppendorf centrifuge and the dried pellets dissolved in SDS-PAGE sample buffer.

For posttranslational membrane association *in vitro* translations were terminated by the addition of 1 mM cycloheximide. 1.2 µl of the *in vitro* translation mixture were further incubated in the presence of ~12 A_{280}/ml salt-washed canine microsomes (RM_{KN}) in a volume of 10 µl at 25° C for 1 h. Carbonate extractions were subsequently performed as described above.

Materials: Radioisotopes were purchased from Amersham, U.K. m^7GpppG and T4-DNA ligase were from Boehringer Mannheim. Restriction endonucleases, and SP6 polymerase were purchased from Genofit, Heidelberg. Cycloheximide was from Sigma, St. Louis.

RESULTS

Docking protein does not assemble into membranes via an SRP/DP-mediated mechanism. The majority of secretory and membrane proteins that are targeted to the rough ER contain N-terminal cleavable signal sequences and are synthesized on polysomes bound to the rough ER (10). For those cases where the experiments have been carried out, such proteins require the participation of the SRP/DP recognition machinery. SRP and DP can also be required for the targeting of proteins that do not have cleavable, or even N-terminal signals (8), and thus the mode of insertion cannot always be deduced from the primary structure.

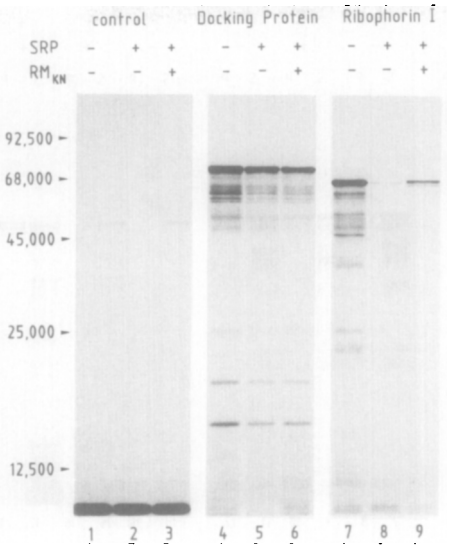

Fig. 1. Translation of DP in a wheat germ cell free lysate is not affected by SRP. Capped transcripts for human DP (lanes 4-6) or human ribophorin I (lanes 7-9) were translated in a wheat germ cell free system in the presence or absence of SRP (40 U/ml) and salt washed rough microsomes as indicated above the figure. Lanes 1-3 represents a translation control of a transcript derived from an pGEM2 construct containing the human DP cDNA insert in the anti-sense direction.

SRP has been shown to arrest the translation of nascent membrane and secretory proteins containing a signal sequence (19). Although translation arrest is limited to the wheat germ system (20), it nonetheless provides a useful assay for the interaction of the nascent chain with SRP. The synthesis of DP, in contrast to ribophorin I (RI), is not blocked by the addition of SRP, nor is its insertion into microsomes promoted by it. cDNAs encoding DP and RI were transcribed *in vitro* and translated in the wheat germ system in the presence of SRP and salt-washed, nuclease-treated pancreatic microsomes (RM_{KN}). In figure 1 it can be seen that the translation of RI was inhibited by SRP in a fashion similar to most other SRP/DP-requiring proteins (compare lanes 4 and 5 with lanes 7 and 8). Addition of RM_{KN} resulted in the conversion of most of the ribophorin I to the processed and glycosylated form (lane 9) indicative of its proper assembly into pancreatic microsomes.

The lack of inhibition of synthesis by SRP is not sufficient proof of an SRP-independent mode of membrane insertion, as some proteins have been shown to require SRP even though a translation arrest was not observed (21). Several laboratories have reported that DP is an integral membrane protein. This conclusion was based on the fact that the solubilization of DP requires detergents (3,22) and that it is quantitatively associated with the insoluble membranous fraction following carbonate (pH11) washing (18). We therefore used resistance to washing with 0.1M carbonate (pH 11) as a criterion for SRP-dependent membrane insertion, and examined DP and ribophorins I and II (RI and RII). As can be seen in figure 2, RI and RII become inserted, glycosylated and stably associated with rough microsomes in a pH 11-resistant fashion only when SRP was present in addition to RM_{KN} (lanes 7-18). In contrast, DP failed to become associated with the microsomes with or without SRP (lanes 1-6). It was thus obvious that DP does not contain the functional equivalent of a signal sequence, and that it must become associated with microsomes via a mechanism exclusive of itself and SRP.

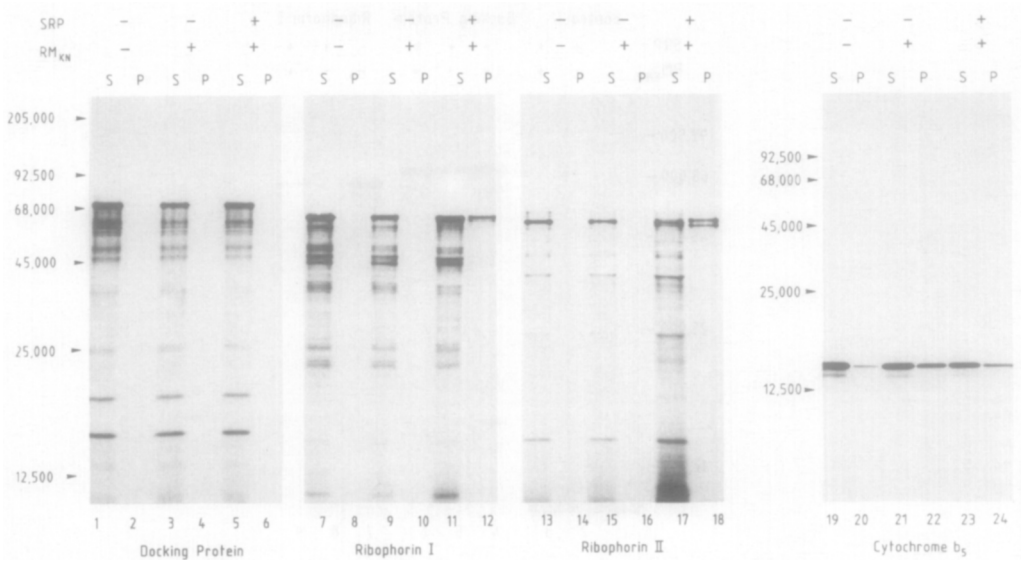

Fig. 2. DP does not become membrane associated by a signal or insertion sequence mediated mechanism. *In vitro* transcripts coding for human DP (lanes 1 to 6), human ribophorin I (lanes 7 to 12), human ribophorin II (lanes 13 to 18), or rat cytochrome b_5 (lanes 19 to 24) were translated in the absence or presence of salt washed canine microsomes (RM_{KN}) and SRP. An aliquot of the translation mixture was extracted with 0.1 M Na_2CO_3, pH 11, and supernatant (S) and pellet (P) fractions were analyzed by fluorography.

DP does not assemble into membranes via a hydrophobic insertion sequence. A certain set of proteins of the ER assemble spontaneously into membranes in an SRP-independent fashion via a hydrophobic insertion sequence (10). Cytochrome b_5 is an example of such a protein (21,23), having a hydrophobic tail on its C-terminus (24). As can be seen in figure 2 (lanes 19-24), cytochrome b_5 was able to become associated with RM_{KN} in a pH 11-resistant fashion regardless of the presence of SRP. When the biogenesis of DP was examined under the same conditions, no such insertion was found to take place (fig. 2, lanes 1-6). Taken together, these data indicate that DP must assemble into microsomes via an as-yet-uncharacterized mechanism, one that has not been observed for any sort of protein to date.

Assembly of DP into microsomes *in vitro* may be receptor-mediated. The possibility exists that DP becomes associated with the ER membrane via interaction with another membrane protein. Tajima *et al.* (9) have reported that DP interacts, *in situ*, with a smaller polypeptide of 30 kDa. They have also shown that this 30 kDa "ß-subunit" of DP is present in membranes in a slight excess over the 73 kDa species. The ideal experiment would be to either load up rough microsomes with ß-subunit or to deplete them of the 73 kDa subunit and then use such vesicles to study the membrane association of DP. Unfortunately such approaches are not yet possible. On the one hand, the β-subunit has not been cloned, and "loading" microsomes would therefore not be possible *in vitro*. On the other, the existing pool of 73 kDa subunit cannot be removed from the membrane under non-denaturing conditions (9). The fact that the ß-subunit is present in stoichiometrically greater amounts in rough microsomes compared to 73 kDa DP implies, however, that normal microsomes, if present in sufficient quantity, should allow at least some DP to become inserted into the membrane *in vitro* in a pH 11-resistant fashion.

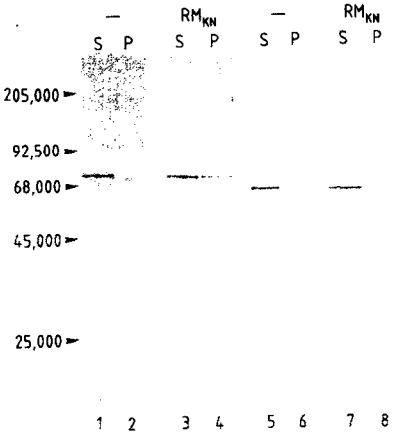

Fig. 3. Post-translational association of human DP with salt-washed microsomal membranes. Human DP (lanes 1-4) or human ribophorin I (lanes 5-8) transcripts were translated for 1 h at 25° C in a wheat germ cell free system. After termination of translation with cycloheximide a small aliquot was incubated in the absence (lanes 1, 2, 5 and 6) or presence (lanes 3, 4, 7 and 8) of microsomal membranes (RM_{KN}) for 1 h at 25° C. Carbonate extractions, SDS-PAGE, and fluorography was carried out as described in the Material and Methods section.

Accordingly, we set up a post-translational assay, whereby reduced amounts of DP or RI (10% of those used in fig. 2) were incubated with higher than usual amounts of microsomes. In this situation, where the unoccupied putative receptor (the ß-subunit) is present in sufficient quantity, a stable (pH 11-resistant) association of 73 kDa DP with the membrane did occur (fig. 3, lane 4). Under these conditions, where a high ratio of membrane to translation product existed, such a result may have occurred by trapping or non-specific membrane association. This was shown not to be the case, as ribophorin I, under the same conditions, was not present in sedimentable material after carbonate washing (lane 8). This result would confirm the existence of a limited quantity of an assembly-mediating component in the microsomal membrane whose stoichiometry approximates that of the previously reported ß-subunit (9).

The amount of 73 kDa DP that was assembled into the microsomal vesicles showed an excellent correlation with the predicted amount of ß-subunit already present. Based on the quantitation of Tajima *et al.* (9), 33.6 fmol of free ß-subunit existed in the aliquot of membranes used in the study whose results are shown in fig. 3. Using standard procedures, one can calculate that the amount of 73 kDa DP present in our assay corresponded to 111 fmol. By scanning autoradiograms (fig. 3) we determined that 25-35% of the DP in the assay became stably associated with the microsomes. Thus we can conclude that 28-39 fmol of 73 kDa DP was assembled. These data lend strong support to the notion that the biosynthetic association of DP with microsomal membranes occurs via an interaction with the ß-subunit. Moreover, this assembly can take place efficiently *in vitro*, in a post-translational fashion, involving most of the free ß-subunit.

DISCUSSION

In this report we have examined the biogenesis of the human DP. Our results indicate that the docking protein does not directly avail itself of the targeting machinery of which it is an integral part in order to enable its own integration into the membrane of the rough ER, nor does it make use of a hydrophobic insertion sequence. Instead, our data support, but by no means prove, the hypothesis that DP is assembled in conjunction with another membrane protein.

Previous studies have shown that 59 out of 73 kDa of DP can be removed in an active state from the membrane by mild proteolysis (16). Accordingly, the conclusion has been drawn that the membrane anchoring domain of this protein resides in the N-terminal 14 kDa (6,18). That DP could be an integral membrane protein was deduced from the fact that it can only be isolated from rough microsomes in intact form by detergent extraction (2,3), that it partitions into the cloud-point precipitates of Triton X-114 (Meyer and Hortsch, unpublished) and that it is resistant to washing with carbonate at pH 11 (18). The primary sequence data for both the human and canine forms of DP is consistent with the notion that DP might be inserted into the lipid bilayer by virtue of one or both of the hydrophobic sequences at its amino terminus (residues 4-22 and 62-79) (6,7).

Several lines of evidence, including the data presented here, speak against a direct insertion by virtue of these sequences. First of all both the canine and the human DP have lysines at positions 10 and 72 interrupting the uncharged nature of the putative insertion sequence. *In vitro* experiments, using cytochrome b_5 as a control, show that DP does not become associated with microsomes in this fashion. Furthermore, when microsomes are labeled with the photoactivated hydrophobic affinity probe TID (25), DP does not become labeled, whereas other rough ER-specific integral membrane proteins, such as both ribophorins, do (Hortsch and Meyer, unpublished). Lastly, DP associates strongly, under non-denaturing conditions, with another integral membrane protein of 30 kDa (9) that may in fact represent the bona fide anchor for DP, or at least facilitate DP's association with the membrane.

Recent studies have shown several membrane proteins to be anchored in membranes by fatty acids, phospho- or glycolipids (26). This possibility is unlikely in the case of DP as lipids are one of the primary targets of TID (25), and thus one would expect DP to be labeled. Additionally, lipid-anchored proteins have been found primarily associated with the plasma membrane of cells. Moreover, DP does not have a blocked amino terminus (6), a typical feature of myristilation.

Our results are most consistent with a receptor-mediated form of assembly. When a small amount of DP was presented to microsomes, where a slight excess of ß-subunit has been shown to be present, membrane integration into a pH 11-resistant form was observed. From this experiment one cannot easily extrapolate to the in vivo situation. In vivo one can envision that either the 73 and the 30 kDa subunits are synthesized coordinately and oligomerize prior to assembly into the rough ER, or that the 30 kDa subunit is assembled first possibly via an SRP/DP dependent mechanism and serves as a receptor for the 73 kDa subunit. It will in future be possible to discriminate between these alternatives using *in vitro* assay systems. Such experiments can be carried out as soon as a full length clone for the 30 kDa ß-subunit becomes available.

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