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A tetrameric complex of membrane proteins in the endoplasmic reticulum

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The translocation site (translocon), at which nascent polypeptides pass through the endoplasmic reticulum membrane, contains a component previously called 'signal sequence receptor' that is now renamed as 'translocon-associated protein' (TRAP). Two glycosylated subunits of the TRAP complex have been identified before (α and β subunits). We now show that the TRAP complex is actually comprised of four membrane proteins (α, β, γ, δ), present in a stoichiometric relation, which are genuine neighbours in intact microsomes. The amino acid sequences of the additional, non-glycosylated subunits were deduced from cloning of the corresponding cDNAs. The δ subunit spans the membrane only once and has its major portion, containing a disulfide bridge, at the lumenal side. The γ subunit is predicted to span the membrane four times.

Proteins are translocated across the endoplasmic reticulum (ER) membrane at specific sites (translocons [1]), probably through protein-conducting channels (for review see [2]). To identify membrane proteins located in the vicinity of translocating polypeptides, crosslinking methods have been applied [3–9]. Short translocating polypeptide chains, representing an early phase of the translocation process, can be crosslinked through their signal sequence to an integral, glycosylated membrane protein that is about 35 kDa and has a cytoplasmic tail of about 5 kDa [3]. Based on these properties, the 'signal sequence receptor' (α subunit) was purified [10] and received its name on the assumption that it was identical with the major crosslinking partner of short nascent chains. However, it is now clear that both this protein and the recently discovered 'translocating-chain-associating membrane' (TRAM) protein [8] meet the predicted properties and that the TRAM protein is actually the major crosslinking partner [8]. The 'signal sequence receptor' is most likely not even required for the translocation process: proteoliposomes reconstituted from a detergent extract that had been immunodepleted from it [11], or proteoliposomes containing the TRAM protein as the only glycoprotein [8], showed unimpaired translocation activity.

We have therefore decided to change the name of the 'signal sequence receptor' to 'translocon-associated protein' (TRAP) since the protein, although not a signal sequence receptor, seems to be located at the translocation site. The α subunit of TRAP (TRAPα) can be crosslinked to a minor extent to various translocating chains, and the proportion of TRAPα among glycoprotein crosslinks appears to increase as chain length increases [8]. TRAPα is segregated to the rough portion of the ER [12], it can be crosslinked to membrane-bound ribosomes [13] and TRAP is associated in part with ribosomes after solubilization of rough microsomes with detergents [9]. The protein is a major constituent of the ER membrane of various species [10]. Finally, antibodies against TRAPα and Fab fragments prepared from the antibodies inhibit the in vitro translocation of several secretory proteins [10].

The amino acid sequence of TRAPα was deduced from cloning of its cDNA and indicates that it is a single-spanning membrane protein [14]. Employing Nonidet P-40 as a detergent to solubilize canine pancreatic microsomes, it was found that TRAPα is tightly and stoichiometrically associated with a second protein, called TRAPβ (previously SSRβ) [6]. The cDNA of TRAPβ has also been cloned and codes for a glycosylated, single-spanning membrane protein [6].

In the present study, we have used digitonin as a milder detergent to solubilize the membranes and show that there are two further subunits in the TRAP complex (γ and δ). The four subunits are genuine neighbours in the ER membrane, as indicated by crosslinking with a bifunctional reagent. Using cDNA cloning, we have determined the primary structures of the additional subunits and propose a topological model for the TRAP complex.

MATERIALS AND METHODS

Antibodies

Antibodies to a peptide comprising the C-terminal 15 amino acids of TRAPα have been characterized before [6, 14].

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Abbreviations. ER, endoplasmic reticulum; TRAM protein, translocating-chain-associating membrane protein; TRAP, translocon-associated protein.

Note. The novel DNA sequence data for TRAPγ (previously SSRγ) and TRAPδ (previously SSRδ) published here have been deposited with the EMBL sequence databank and are available under the accession numbers RNSSRGAM Z14030 and RNSSRDSUB Z19087, respectively.

They were affinity purified and immobilized as described [6]; 1 ml affinity resin contained about 3 mg immunoglobulin.

Antibodies against the complex of TRAPα and TRAPβ were raised using preformed immunocomplexes as follows. 100 µg of the complex, purified from a Nonidet P-40 extract of canine microsomes [6], was mixed with 100 µg affinity-purified antibodies directed against the C-terminus of TRAPα. Rabbits were immunized three times with these immunocomplexes. The antibodies were affinity-purified on an Affigel column to which the complex of TRAPα and TRAPβ had been coupled [15]. The antibodies were then bound to protein-A−Sepharose and crosslinked with dimethyl suberimidate [15]. Before using the column, a mock elution with 0.1 M glycine/HCl pH 2.2 was performed.

Purification of the TRAP complex

Rough microsomes from dog pancreas were prepared according to standard procedures [16]. Purification of the complex of TRAPα and TRAPβ was carried out by immunoaffinity chromatography after solubilization of the membranes in Nonidet P-40, as described [6].

Purification of the tetrameric TRAP complex was carried out as follows. 6000 equivalents (eq.; for definition, see [17]) rough microsomes were solubilized in 12 ml 50 mM Hepes/KOH pH 7.6, 600 mM potassium acetate, 3% digitonin, 10% glycerol, 1 mg/ml each of leupeptin, pepstatin, elastatinal and chymostatin. After centrifugation to pellet ribosomes and particles with sedimentation coefficients larger than 30S, the extract was passed with a flow rate of about 1−2 ml/h over a 1-ml column containing immobilized antibodies against TRAPα. The column was washed with 50 vol. 50 mM Hepes/KOH pH 7.6, 500 mM potassium acetate, 0.1% digitonin. Elution of the complex was carried out with 0.1 M glycine/HCl pH 2.2, 0.2% Nonidet P-40. The eluted proteins were precipitated with 10% trichloroacetic acid, the pellet was washed with acetone and dissolved in SDS-sample buffer.

Purification of the TRAP complex after protease treatment of microsomes

Rough microsomes were incubated with a mixture of 50 µg/ml each of trypsin and chymotrypsin for 1 h on ice. The reaction was stopped by adding in series 100 µg/ml N-p-tosyl-L-lysine chloromethane, 200 µg/ml N-tosyl-L-phenylanaline chloromethane, 1 mM phenylmethylsulfonyl fluoride and 10 µg/ml aprotinin (final concentrations). After solubilization of the membranes with digitonin, the digestion products of TRAP were purified by immunoaffinity chromatography. Immobilized, affinity-purified antibodies were used that were raised against the complex of TRAPα and TRAPβ (see above) and that are primarily directed against lumenal domains of these polypeptides. Proteins bound to the antibody column were eluted with 0.1 M glycine/HCl pH 2.2 and analyzed by SDS-gel electrophoresis.

Purification of TRAPγ and TRAPδ

The tetrameric TRAP complex, after solubilization with digitonin, was bound to an antibody column as described above. After washing, TRAPγ was dissociated from the complex with 50 mM Hepes/KOH pH 7.6, 700 mM potassium acetate, 2% Nonidet P-40. The remaining subunits were eluted with 0.2 M glycine/HCl pH 2.2, 0.1% Nonidet P-40 and submitted to preparative SDS-gel electrophoresis. The δ subunit was eluted by shaking the gel slice in 1% SDS.

Amino acid sequencing

The N-terminal sequences were determined directly by automated sequencing. TRAPγ was submitted to cleavage by the protease Asp-N (Boehringer Mannheim) or by cyanogen bromide. TRAPδ was cleaved with trypsin or Glu-C (Boehringer Mannheim). The resulting peptides were separated by reverse-phase HPLC. Some were subjected to automated sequencing (see Figs 2 and 3).

cDNA cloning

Degenerate oligonucleotides were synthesized corresponding to the peptides KQQSEED (N-terminus of TRAPγ), VEPQITP (N-terminus of TRAPδ) and HAGTYEV (part of a tryptic peptide of TRAPδ). These were used to screen a cDNA library prepared from mRNA of rat liver polysomes which was reverse-transcribed into cDNA by random priming. Labelling of oligonucleotides and screening were performed as described [14]. Positive clones were sequenced by the dideoxy method. In the coding region the sequence of both DNA strands was determined.

Miscellaneous techniques

Crosslinking and diagonal electrophoresis were performed as described before [6]. SDS-gel electrophoresis was carried out according to Laemmli [18] using 12.5% acrylamide gels.

RESULTS

The TRAP complex comprises four polypeptides

For purification of the TRAP complex, immuno-affinity chromatography was used [6]. Rough microsomes from dog pancreas were solubilized in either Nonidet P-40 or digitonin. After centrifugation to pellet ribosomes and membrane remnants, the detergent extracts were passed over columns containing immobilized, affinity-purified antibodies directed against the C-terminus of TRAPα. Elution of the bound material was carried out under acid conditions. Whereas with Nonidet P-40 the previously identified α and β subunits of the TRAP complex were observed (Fig. 1a, lane 1) [6], two additional smaller bands were seen if the microsomes had been solubilized with digitonin (lane 2). The two novel polypeptides of 20 and 18 kDa are called TRAPγ and TRAPδ, respectively. The four TRAP subunits were eluted in approximately equivalent amounts, as judged from their intensity of staining with Coomassie blue. A similar complex was isolated if immobilized antibodies directed against the C-terminus of TRAPβ were used (data not shown).

To test whether the TRAP complex of four polypeptides is genuine, intact microsomes were incubated with the bifunctional reagent dithiobis(succinimidyl propionate) that

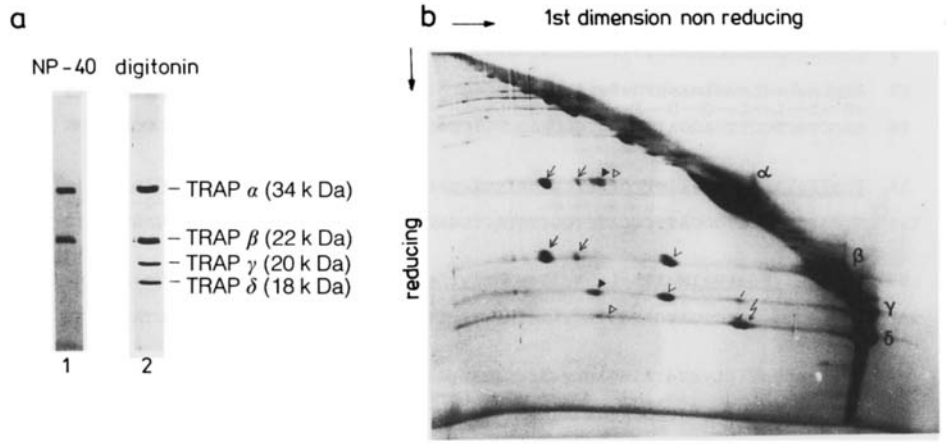

Fig. 1. The TRAP complex consists of four polypeptides. (a) Analysis of the TRAP complex by immuno-affinity chromatography with antibodies directed against TRAPα. Rough microsomes were solubilized with Nonidet P-40 or digitonin and the extracts were passed over an antibody column. Bound material was eluted under acid conditions and analyzed by SDS-gel electrophoresis and staining with Coomassie blue. (b) Determination of next neighbors in intact membranes by crosslinking. Rough microsomes were treated with dithiobis (succinimidyl propionate) and the digitonin-solubilized products, retained by an TRAPα-antibody column, were analyzed by diagonal electrophoresis followed by staining with Coomassie blue. The same type of arrow is used to indicate corresponding constituents of a crosslinked product. The indicated crosslinks between the α and δ and between the γ and δ subunits must be considered to be tentative. The flash sign indicates a homodimer of TRAPδ.

contains a cleavable disulfide bridge. The microsomes were then solubilized with digitonin and, after centrifugation, the crosslinked products were purified by immuno-affinity chromatography with immobilized TRAPα antibodies. The bound material was eluted and analyzed by diagonal electrophoresis (first dimension non-reducing, second dimension after reduction with dithiothreitol; Fig. 1b). Crosslinked products, seen outside the diagonal, were most prominent between the TRAP subunits α and β, α and γ as well as between β and γ (the same type of arrow is used to indicate the corresponding constituents of a crosslinked product). Two crosslinked products were seen for the α and β subunits (diagonal arrows), probably caused by different linkages between the two molecules. Possibly, weak crosslinks between the subunits α and δ (open triangles) and between γ and δ (diagonal slashes) may have also been produced, whereas no crosslinks between the β and δ subunits could be seen. A homodimer of TRAPδ was also found (highlighted by the flash sign), as indicated by the size of the product and by the lack of any other crosslinking partner. We conclude from these results that the four polypeptides of the TRAP complex are genuine neighbors in the ER membrane.

Cloning of the cDNAs for TRAPγ and TRAPδ

To obtain partial amino acid sequences for TRAPγ and TRAPδ, the tetrameric TRAP complex was bound to an antibody column and the γ subunit was selectively eluted by changing the detergent from digitonin to Nonidet P-40. The other three subunits were eluted at acid pH and the δ subunit was further purified by preparative SDS-gel electrophoresis. The separated polypeptides were either subjected directly to N-terminal automated sequencing or they were cleaved by proteases or cyanide bromide, and the resulting peptides were separated by HPLC before sequencing. The amino acid sequences determined are given in parenthesis in Figs 2 and 3 (in single-letter code, except where deviations from the sequence deduced from the cDNA sequence were found). The amino acid sequences of large parts of both polypeptides could be determined. Degenerate oligonucleotides corresponding to selected regions of the partial protein sequences were used to screen a cDNA library from rat liver. The nucleotide sequences of the cDNAs coding for TRAPγ and TRAPδ are given in Figs 2 and 3, respectively. The deduced amino acid sequences are generally in agreement with those determined directly, with some exceptions. The detected amino acid exchanges most likely reflect species differences (dog versus rat) and are often conservative changes.

Comparison of the amino acid sequences deduced from the nucleotide sequences with those determined directly indicates that TRAPδ has a cleavable signal sequence, whereas TRAPγ does not. The 23-residue signal sequence of TRAPδ has typical properties, such as a hydrophobic core and a cleavage site conforming to the -1, -3 rule [19]. The assignment of the initiator methionines is further supported by the occurrence of good translation initiation nucleotide sequences (Kozak sequences; indicated in bold face) [20]. The predicted molecular masses of the TRAP proteins (20988 Da for TRAPγ and 16796 Da for TRAPδ) correspond roughly to those determined by SDS-gel electrophoresis.

The data base does not contain any protein related to TRAPδ. However, for TRAPγ an hitherto unidentified sequence, clearly homologous to it, was found in a randomly sequenced cDNA library of the worm *Caenorhapditis elegans* (EMBL accession number M80055; clone WEST00592) [21]. In the region of the gene where the nucleotide sequence has been unambiguously determined, 49 out of 130 deduced amino acids are identical with those of TRAPγ.

Membrane topologies of TRAPγ and TRAPδ

TRAPδ contains a single hydrophobic sequence close to its C-terminus which is predicted to span the membrane; TRAPγ probably spans the membrane four times (Figs 2–4). Several of the predicted membrane-spanning segments of TRAPγ contain hydrophilic residues, particularly amino acids with hydroxyl groups.

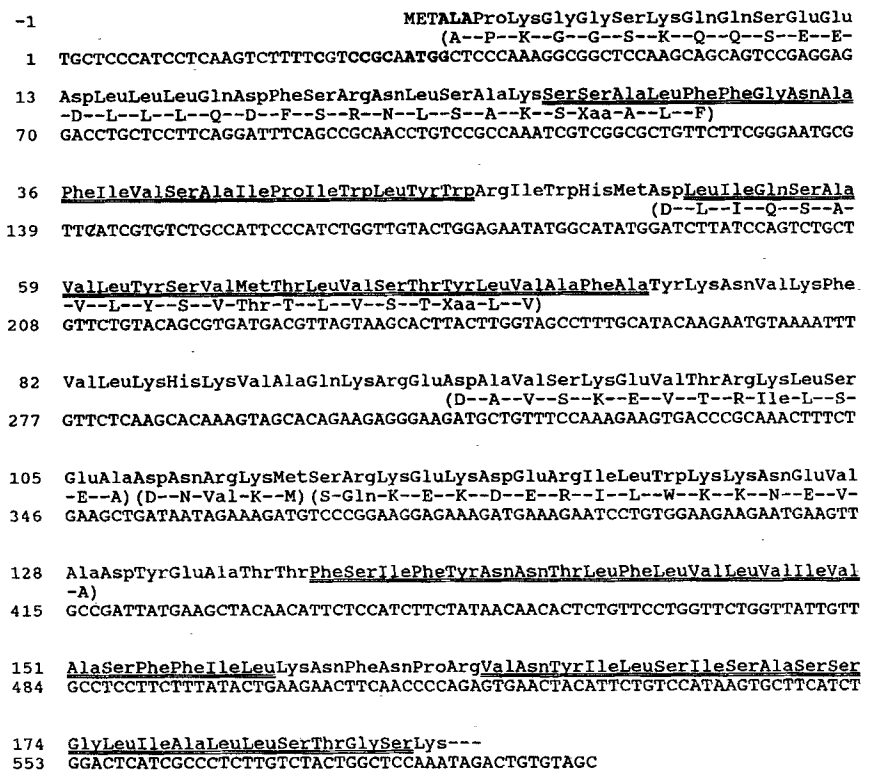

Fig. 2. TRAPγ cDNA sequence and deduced amino acid sequence. cDNA cloning from a rat liver library was performed with degenerate oligonucleotides corresponding to amino acid sequences determined directly. Nucleotides shown in bold face correspond to the Kozak consensus sequence of translation initiation sites [20]. The amino acid sequences determined directly for various peptides derived from dog pancreatic TRAPγ are shown in parenthesis below the amino acid sequence derived from the nucleotide sequence of the cDNA. The single-letter code is used except at positions where the sequences differ. The Ala residue after the initiatior methionine corresponds to the N-terminus of the mature protein. Doubly underlined amino acid sequences are predicted to span the membrane.

TRAPδ contains a disulfide bridge. If the TRAP complex is subjected to SDS-gel electrophoresis under reducing and non-reducing conditions, the only polypeptide that changes its mobility is TRAPδ (Fig. 5a). This result places its N-terminus, containing the only cysteine residues at positions 3 and 34 in the mature protein, into the lumen of the ER, where disulfide bridge formation generally occurs.

Further support for the proposed membrane topologies of the TRAP subunits (Fig. 4) comes from proteolysis experiments. Microsomes were treated with proteases and subsequently solubilized with digitonin. The extract was passed over a TRAP-antibody column containing antibodies directed primarily against the lumenal domains of TRAPα and TRAPβ. The bound fraction was analyzed by SDS-gel electrophoresis (Fig. 5b). As reported previously, proteolysis removes the entire cytoplasmic tail of TRAPα of about 5 kDa [14] but an intermediate was also seen. TRAPβ only looses about 12 amino acid residues at its C-terminus [6]. Similarly, the mobility of TRAPδ changed only slightly if at all, in agreement with the assumption that only eight residues are located in the cytoplasm (see Fig. 3). TRAPγ completely disappeared. Instead, four bands were seen below TRAPδ which seem to be derived from it, but they cannot be the final cleavage products (the sum of their sizes exceeds that of TRAPγ). If the observed products are derived from TRAPγ, the results would be most consistent with the topology shown in Fig. 4 in which the charged segment between the first two and the last two membrane-spanning regions is assumed to be located in the lumen of the ER: partial digestion of the protein at both sides of the cytoplasmic segments would yield products of about the size experimentally observed, whereas the alternative orientation would be expected to yield smaller proteolytic fragments. However, the latter cannot be completely excluded as yet.

DISCUSSION

We report here that the TRAP complex (previously called 'signal sequence receptor' (SSR) complex) consists of four membrane proteins in stoichiometric relation. In addition to the previously identified α and β subunits [6], two further constituents (γ and δ) were found, their sequences were determined and their membrane topology predicted. Crosslinking experiments with a bifunctional reagent indicate that the four subunits are genuine neighbors in intact ER membranes. The fact that a homodimer of TRAPδ was observed suggests that at least two copies of the TRAP complex may come close to each other in the membrane.

The TRAP complex is stable in mild detergents such as digitonin, cholate [11] or BigCHAP (not shown), and it can be isolated by immunoaffinity chromatography with antibodies against TRAPα or TRAPβ or by convential ion-exchange chromatography (data not shown). The interaction of TRAPα with TRAPβ is much stronger than that between them and TRAPγ or TRAPδ; the latter can be readily dissociated by stronger detergents, such as Nonidet P-40. The constituents of the TRAP complex interact with each other either within the membrane or in the lumenal space of the ER, as indicated by the fact that proteolytic removal of the

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  1      GGAAACGGACACGTCAGTGCCGCCAGCGACGTCACCATGCCCGACCCAGCCCGCCGACGCACGATT

 65    GGGTGCTGAGGCCGCTTACGCGTCGCGCTTCCTCGTCTGCTCCGCATGTTCAGGGCGGCTTGCTCTCTTT

-23                                 METAlaAlaMetAlaSerPheGlyAlaLeuAlaLeuLeuLeuLeuSer
134    TCCCCTCGGCAGAGAAGAGGCGATGGCGGCGATGGCATCTTTCGGCGCCCTGGCGCTACTCCTGCTGTCC

- 6    GlyLeuSerCysCysSerAlaGLUAlaCysLeuGluProGlnIleThrProSerTyrTyrThrThrSer
                              (E--A-XaaVal-E--P--Q--I--T--P--S--Y--Y--T--T)
203    GGCCTATCTTGCTGCTCAGCAGAGGCCTGCCTGGAACCCCAGATCACCCCTTCTTACTATACAACTTCA

 17    AspAlaValIleSerThrGluThrValPheIleValGluIleSerLeuThrCysLysAsnArgValGln
272    GATGCCGTCATTTCTACAGAGACTGTATTCATCGTGGAGATCTCACTGACCTGCAAAAACAGGGTCCAG

 40    AsnMetAlaLeuTyrAlaAspValSerGlyLysGlnPheProValThrArgGlyGlnAspValGlyArg
                                                         (G--Q--D--V--G--R-
341    AATATGGCTCTTTATGCCGACGTTAGTGGAAAACAATTTCCTGTAACCCGCGGCCAGGATGTGGGTCGA

 63    TyrGlnValSerTrpSerLeuGluHisLysSerAlaHisAlaGlyThrTyrGluValArgPhePheAsp
       -Y--Q--V--S--W--S--L-AspXaa-K)(S--A--H--A--G--T--Y--E--V--R)(F--F--D-
410    TATCAGGTGTCCTGGAGCCTGGAGCACAAGAGCGCCCATGCAGGCACCTATGAGGTCAGATTCTTCGAT

 86    GluGluSerTyrSerLeuLeuArgLysAlaGlnArgAsnAsnGluAspValSerIleIleProProLeu
       -E--E--S--Y--S--L--L--R)(K--A--Q--R)(N--N--E-XaaIle-S--I--I--P--P--L-
479    GAAGAGTCCTACAGCCTCCTAAGGAAGGCTCAAAGAAATAATGAAGACGTTTCCATCATCCCACCACTG

109    PheThrValSerValAspHisArgGlyThrTrpAsnGlyProTrpValSerThrGluValLeuAlaAla
       -F--T--V--S--V-Xaa-H-R)
548    TTCACAGTCAGTGTGGACCATCGGGGTACCTGGAATGGGCCTTGGGTCTCCACGGAGGTGCTGGCTGCA

132    AlaIleGlyIleValIleTyrTyrLeuAlaPheSerAlaLysSerHisIleGlnAla---
617    GCAATTGGCATAGTGATCTACTACCTAGCTTTCAGTGCAAAGAGCCACATCCAGGCCTGAGGGCAGCAAC

686    CTCAGCCCTCCATTGCTTCTTTCAATAAACAGTCACTATTTGAAAAAAAAAAAAAAAAAAAAAAAAA
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Fig. 3. TRAPδ cDNA sequence and deduced amino acid sequence. cDNA cloning from a rat liver library was performed as for TRAPγ (see Fig. 2). The labelling is the same as in Fig. 2. The singly underlined sequence corresponds to the cleavable signal sequence of TRAPδ. The Glu residue in bold face is the N-terminal residue of the mature protein. The disulfide bridge between Cys3 and Cys34 is indicated.

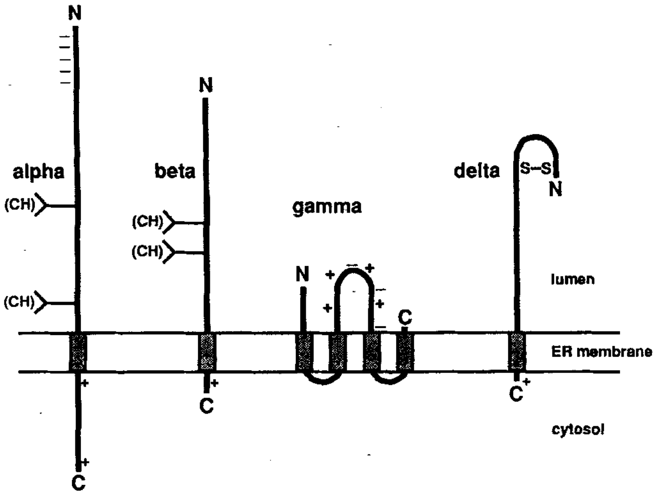

Fig. 4. Proposed topological models for the subunits of the TRAP complex. Evidence for the topologies of TRAPα and TRAPβ has been provided before [6, 14], those for TRAPγ and TRAPδ are based on the amino acid sequences and on the data shown in Fig. 5 of this paper. Negatively and positively charged regions of the proteins are indicated in a schematic manner. (CH), Asn-linked carbohydrate chain.

cytoplasmic domains does not lead to the dissociation of the complex.

Using antibodies directed against TRAPα, Migliaccio et al. [11] have also recently reported the isolation of a TRAP complex consisting of four subunits. The N-terminal amino acid sequences determined by them for TRAPγ and TRAPδ are in complete agreement with our data. In contrast, Wada et al. [22] have described a complex comprising, in addition to TRAPα and TRAPβ, proteins of 25 kDa and 90 kDa. We have not observed these polypeptides with a variety of purification procedures. It seems possible that the special conditions employed by Wada et al. [22] lead to a different complex and/or to insufficient removal of contaminating polypeptides.

It should be noted that some of the TRAP complex sediments with the ribosomes after solubilization of rough microsomes [9]. This population of TRAP may be bound either directly to ribosomes or via one or more ribosome-associated membrane proteins.

We now know the primary structures of all subunits of the TRAP complex and have derived topological models for them (Fig. 4). Three subunits are single-spanning membrane proteins (α, β, δ), TRAPγ spans the membrane probably four times. TRAPα and TRAPβ are glycoproteins, each carrying two carbohydrate chains; TRAPδ contains a disulfide bridge. None of the subunits has a sizable cytoplasmic domain. TRAPα has a highly negatively charged region at its N-terminus, which has been suggested to be involved in Ca^{2+}-binding [22]. The negative charges, but not the precise amino acid sequence in this region, are conserved among TRAPα proteins from dog, human and trout. In other parts of TRAPα, the sequences of the various species are very similar (our unpublished results). TRAPγ has a charged domain between the first two and the last two membrane-spanning segments which may be responsible for the interaction with the other

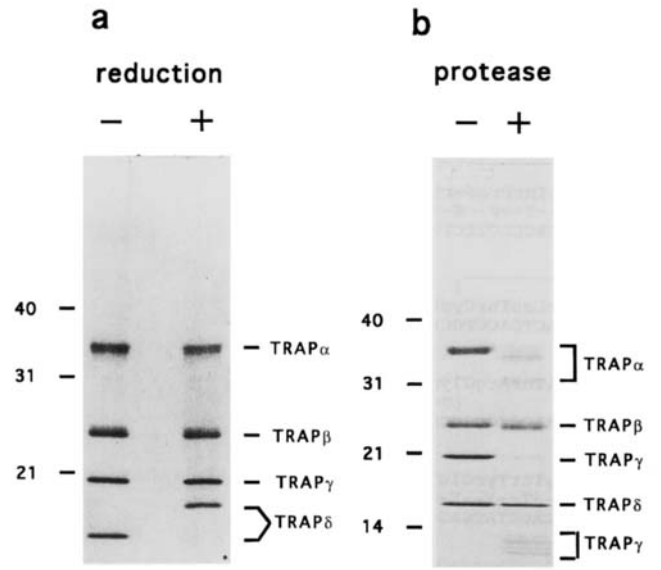

Fig. 5. Experimental evidence for the topology of the TRAP subunits. (a) TRAPδ has a disulfide bridge. The tetrameric TRAP complex was submitted to SDS-gel electrophoresis under nonreducing or reducing conditions and the proteins were visualized by staining with Coomassie blue. (b) Cleavage of cytoplasmic domains by proteases. Rough microsomes were treated with trypsin and chymotrypsin and the digestions products of the TRAP complex were purified by immuno-affinity chromatography using antibodies directed primarily against the lumenal domains of TRAPα and TRAPβ. The proteins were analyzed by SDS-gel electrophoresis followed by staining with Coomassie blue. Numbers on the left are molecular masses of marker proteins in kDa.

subunits of the TRAP complex. The related gene product of *C. elegans*, found in the data base [21], has a particular similarity in the charged domain and probably shares the predicted membrane topology with TRAPγ. It seems likely that the protein represents the TRAPγ homolog in this lower organism.

The function of the TRAP complex is, as yet, obscure. Several possibilities come to mind. It may function in the retention of ER proteins [22], be involved in the translocation of a subclass of proteins or may serve as a membrane chaperone, facilitating the folding of multi-spanning membrane proteins or the assembly of membrane protein complexes. It is also conceivable that TRAP has an unidentified enzymatic activity required for the modification of nascent polypeptides. It is indeed interesting to note several properties that the TRAP complex shares with the oligosaccharyl transferase [23–25]: both are abundant and consist of different membrane protein subunits that have their major portions in the lumen of the ER, both are associated with ribosomes and antibodies directed against them have similar effects on the translocation process. Whatever the function of the TRAP complex may be, it seems to be highly conserved during evolution.

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