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Transcytosis-associated protein (TAP)/p115 is a general fusion factor required for binding of vesicles to acceptor membranes

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ABSTRACT **Transcytosis-associated protein (TAP) is found on transcytotic vesicles (TCVs) and is required for their fusion with the target membrane. We developed a cell-free assay capable of differentiating targeting/binding of TCVs to membrane from later fusion events. We found that TAP mediates stable association of TCVs with the target membrane. The sequence of rat liver TAP (959-amino acid open reading frame) encodes a protein that contains (*i*) an N-terminal region (amino acids 1–649), (*ii*) an internal region with several coiled-coil stretches (amino acids 650–930), and (*iii*) a C-terminal acidic region (amino acids 931–959). Comparisons between TAP and other sequences indicate that TAP is identical to p115, a protein involved in *cis* to *medial* Golgi transport, and homologous to Uso1p, a yeast protein involved in endoplasmic reticulum to Golgi transport. Our findings suggest that TAP/p115/Usop1 is a general factor acting within the secretory and endocytic pathways to bind transport vesicles prior to membrane fusion.**

Vesicular transport (transcytosis) of proteins from the basolateral to apical plasma membrane (PM) of polarized epithelial cells has been extensively studied (1, 2). We have designed a cell-free assay reconstituting the last step of transcytosis, the fusion of transcytotic vesicles (TCVs) with the apical PM (3–6), and have identified two proteins, *N*-ethylmaleimide-sensitive fusion protein (NSF) and transcytosis-associated protein (TAP), which are required for fusion (3, 7). We now further analyze TAP's role by establishing a modified *in vitro* assay that differentiates between the targeting/binding stage and subsequent steps of the fusion reaction. We show that TAP is required for the binding of TCVs to the PM.

We have cloned and sequenced TAP.† TAP's sequence is identical to that of p115 (8), a protein originally shown to be required for *cis* to *medial* Golgi transport (9) and subsequently for an uncoupled reaction measuring exclusively intra-Golgi fusion (10). TAP also shares highly conserved regions with a yeast protein Uso1p, which among other pleiotropic effects has been implicated in endoplasmic reticulum (ER) to Golgi traffic (11).

MATERIALS AND METHODS

SDS/PAGE and Immunoblotting. Samples were processed for SDS/PAGE and immunoblotting as described (3, 12). Immunoblots were processed by chemiluminescence and filters were exposed to x-ray film.

Cell-Free Assay. *In vivo* radiolabeled donor fractions and unlabeled target fractions and cytosol were prepared from rat livers as described (3). Fusion assays and analysis of polymeric IgA receptor (pIgA-R) were performed as described (3). In some experiments, reaction mixtures were centrifuged, and supernatant and pellets were separated. For the immunodepletion experiments, anti-TAP antibodies were purified from culture supernatants using protein G-Sepharose (Pharmacia) and coupled to Affi-Gel 10 (Bio-Rad) support matrix. TAP-immunodepleted cytosol was prepared by incubating cytosol with 4G2 monoclonal antibodies (mAbs) covalently bound to the Affi-Gel support at 4°C.

TAP Purification. Rat livers were homogenized in 25 mM Tris·HCl, pH 7.4/250 mM sucrose/150 mM KCl/1 mM dithiothreitol. The homogenate was centrifuged, the supernatant was collected, and ammonium sulfate was added to a concentration of 40%. The pellets were recovered, resuspended in TD (25 mM Tris·HCl/1 mM dithiothreitol) and diluted to a conductivity equal to TD containing 150 mM KCl (150KTD). This material was loaded onto a DEAE-Sepharose (Pharmacia) column equilibrated with 150KTD. The column was eluted with a gradient of 150–600KTD. Fractions were analyzed by immunoblotting with polyclonal anti-TAP antibody. A concentrated pool was loaded onto a Superose 6 (Pharmacia) column equilibrated with 150KTD. TAP-containing fractions were pooled and loaded onto a Mono Q (Pharmacia) column equilibrated with 150KTD. The column was eluted with a gradient of 150–600KTD.

mAbs. Mono Q fractions were used as antigen to generate antibodies in mice (13). Screening of hybridomas was performed by immunoblotting. Two cell lines secreting anti-TAP antibodies were used in this study.

Cloning and Sequencing. We used degenerate oligonucleotide primers corresponding to amino acid residues QHDNIVTH (amino acids 647–654) and TQQASQIQ (amino acids 688–695) to amplify a cDNA fragment from reverse-transcribed total liver RNA (SuperScript kit; GIBCO/BRL). The resulting PCR fragment was cloned and sequenced and was 84% homologous to a human partial cDNA sequence (GenBank accession no. Z24991). The fragment was used to screen an oligo(dT)-primed λ ZAP II cDNA library from a rat liver cell line. A 5′-terminal restriction fragment of a partial cDNA clone was used to screen a random-primed rat liver cDNA library in pUEX. A single clone (≈1.6 kb) was isolated that contained the 5′ end of the TAP coding sequence. Standard procedures were used for cloning and sequencing (14).

RESULTS AND DISCUSSION

Anti-TAP mAbs. Previously, we demonstrated that cytosol immunodepleted of TAP failed to support fusion of TCVs with apical PM (3), but these experiments used a polyspecific polyclonal antibody raised against purified TCVs (15). To

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Abbreviations: PM, plasma membrane; TCV, transcytotic vesicle; NSF, *N*-ethylmaleimide-sensitive fusion protein; TAP, transcytosis-associated protein; ER, endoplasmic reticulum; pIgA-R, polymeric IgA receptor; mAb, monoclonal antibody; ORF, open reading frame; SNAP, soluble NSF attachment protein; SNARE, SNAP receptor.

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†The sequence reported in this paper has been deposited in the GenBank data base (accession no. U15589).

unequivocally show that TAP is required for transcytotic fusion, we used mAb immunodepletion to prepare TAP-free cytosol. Such cytosol was then used in the cell-free assay to determine whether it could support fusion and, if not, whether addition of purified TAP could reconstitute fusion.

To test the mAbs, cytosolic proteins were immunoblotted with either the mAbs or the polyclonal anti-TAP antibody. As shown in Fig. 1*A*, for the 5D6 mAb, only TAP was detected (lane 2), while the polyclonal anti-TAP antibody reacted with TAP but also detected other minor proteins (lane 3). To ensure that the mAbs recognize the same protein as the polyclonal anti-TAP antibody, cytosol was subjected to immunoprecipitation with 4G2 and 5D6 mAbs or without antibodies and then immunoblotted with the polyclonal anti-TAP antibody. As shown in Fig. 1*B*, TAP was present in the +mAb immunoprecipitates (lanes 1 and 4) but was absent from the −mAb immunoprecipitate (lane 3). These results indicate that the mAbs immunoprecipitate TAP. The 4G2 mAb was used to immunopurify TAP from cytosol (Fig. 1*C*).

TAP Is Required for Transcytotic Fusion. 4G2 mAb was used to immunodeplete cytosol (removing >95% of TAP), which was then tested in the transcytotic cell-free assay. The donor fraction was prepared from *in vivo* radiolabeled rats and contains TCVs that carry radiolabeled 120-kDa pIgA-R. The target fraction was prepared from unlabeled rats and contains an inside-out apical PM that has an exoprotease capable of cleaving pIgA-R to an ≈90-kDa fragment. When donor and target fractions were mixed with cytosol and an ATP-regenerating system and incubated at 37°C, fusion occurred as measured by the cleavage of 120-kDa pIgA-R to the ≈90-kDa fragment (Fig. 2, lane 1). Under control conditions, 50–80% fusion is routinely observed. A similar result (lane 4) was observed when mock-depleted cytosol was used. However, no fusion was observed (lane 7) when TAP-depleted cytosol was tested. Addition of purified TAP (lane 8) reversed the inhibition to the same extent as addition of untreated cytosol (lane 9). Addition of purified TAP or untreated cytosol to reaction mixtures containing untreated cytosol (lanes 2 and 3) or to reaction mixtures containing mock-depleted cytosol (lanes 5 and 6) did not increase fusion efficiency.

Dissection of the Transcytotic Assay. To address the role of TAP at a more refined level, the cell-free assay was dissected into distinct stages by changing the end-point readout; instead of measuring fusion, we assayed the association of TCVs with the target PM. We developed a velocity centrifugation protocol (based on the large difference in size between the donor TCVs and the target PM), which can separate free TCVs from

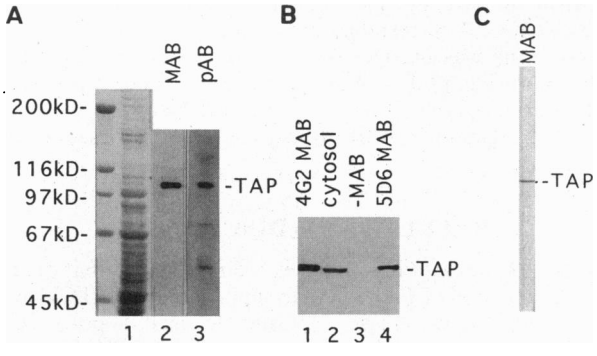

FIG. 1. Characterization of anti-TAP mAbs. (*A*) Rat liver cytosol was processed by SDS/PAGE and stained with Coomassie blue (lane 1) or immunoblotted with 5D6 mAb (lane 2) or with polyclonal anti-TAP antibody (lane 3). (*B*) Cytosol was immunoprecipitated with 4G2 and 5D6 mAbs or without antibody. Immunoprecipitated proteins were immunoblotted with the polyclonal anti-TAP antibody. Cytosol was included to indicate position of TAP. (*C*) 4G2 mAb was used to immunopurify TAP from cytosol. A Coomassie blue-stained SDS/polyacrylamide gel of purified material is shown.

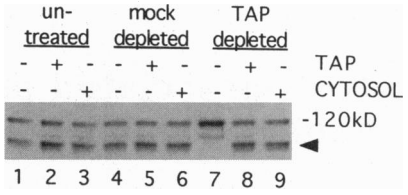

FIG. 2. TAP is required for transcytotic fusion. Fusion reaction mixture containing untreated cytosol, mock-depleted cytosol, or TAP-depleted cytosol were incubated at 37°C for 60 min (lanes 1, 4, and 7) or were supplemented with purified TAP (lanes 2, 5, and 8) or with untreated cytosol (lanes 3, 6, and 9) prior to incubation at 37°C. pIgA-R was immunoprecipitated and analyzed by SDS/PAGE and fluorography.

the target PM. The donor or the target fraction was incubated in a complete reaction mixture at 37°C for 60 min and then centrifuged. The extent of sedimentation of the donor fraction in the absence of the fusing partner was analyzed by examining the resulting pellet and supernatant for the content of the 120-kDa form of pIgA-R. Pelletability of the target fraction in the absence of donor was assayed by examining the pellet and supernatant fractions for content of the apical PM protein dipeptidyl-peptidase IV. As shown in Fig 3*A* (untreated bars), the centrifugation results in minimal (≈10%) pelleting of TCVs, but as shown in Fig. 3*B* (untreated bars), it results in extensive (≈65%) sedimentation of target PM.

Donor or target fractions were supplemented with reaction mixtures containing TAP-depleted cytosol or an ATP-depleting system at 37°C for 60 min and centrifuged. As shown

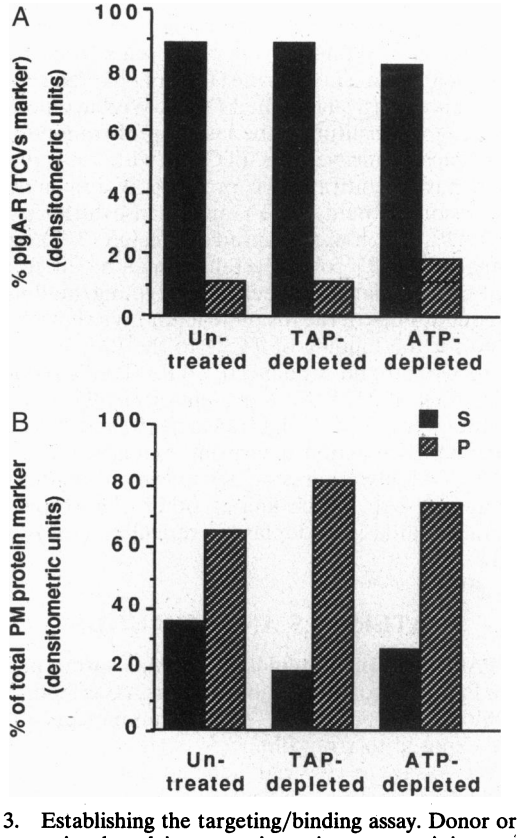

FIG. 3. Establishing the targeting/binding assay. Donor or target fraction was incubated in a reaction mixture containing untreated cytosol (untreated bars), TAP-depleted cytosol (TAP-depleted bars), or untreated cytosol and ATP-depleting system (ATP-depleted bars). Reaction mixtures were incubated at 37°C for 60 min and then separated by centrifugation. (*A*) Amount of pelleted and nonpelleted donor TCVs was assayed by immunoblotting with anti-pIgA-R antibodies. (*B*) Pelleting characteristics of the target PM was determined by immunoblots with anti-dipeptidyl-peptidase IV antibodies.

in Fig. 3*A* (TAP-depleted and ATP-depleted bars), there was no change in the sedimentation characteristics of TCVs. Similarly, as shown in Fig. 3*B* (TAP-depleted and ATP-depleted bars), the pelletability of the target apical PM under such conditions is similar to that seen in the control reaction.

TAP Is Required for Binding of TCVs to Target Membrane. To examine the stage at which TAP is required for transcytotic fusion, we analyzed the centrifugation behavior of three fusion reaction mixtures: (*i*) control reaction mixture (containing donor and acceptor, untreated cytosol, and an ATP-regenerating system); (*ii*) reaction mixture containing TAP-depleted cytosol; and (*iii*) reaction mixture containing an ATP-depleting system. As shown in Fig. 4*A*, fusion occurs in the control (lane 1) reaction mixture but is inhibited in the TAP-depleted (lane 2) and ATP-depleted (lane 3) reaction mixtures. An aliquot of each reaction mixture was subjected to centrifugation and the resulting supernatant and pellet fractions were analyzed by SDS/PAGE and fluorography to determine their content of radiolabeled pIgA-R. As shown in Fig. 4*B* (lanes 1 and 2), ≈82% of pIgA-R present in the control reaction mixture was recovered in the pellet, with the remainder found in the supernatant. The pIgA-R recovered in the pellet consists predominantly of the 90-kDa fragment [which is released into the sealed lumen of the target PM (3) and is therefore pelletable], and low levels of the uncleaved 120-kDa form. The ≈20% of 120-kDa pIgA-R recovered in the pellet is higher than the ≈10% that pellets in the absence of target PM (Fig. 3*A*, untreated bars) and might be due to pIgA-R present in TCVs bound to the PM but not yet fused.

Different results were obtained when a reaction mixture containing TAP-depleted cytosol was incubated at 37°C for 60 min and then subjected to centrifugation. As shown in Fig. 4*B* (lanes 3 and 4), the majority of pIgA-R remained in the supernatant (the ≈10% of pIgA-R found in the pellet is analogous to the background level seen in Fig. 3*A*, untreated bars). All of the pIgA-R was in the 120-kDa form. These results suggest that TAP is required for binding of TCVs to the target PM or, alternatively, that the centrifugation conditions are too stringent and dissociate bound TCVs from the target PM if fusion is prevented. To distinguish between these possibilities, we examined the distribution of pIgA-R when an ATP-depleted reaction mixture was centrifuged (16). As shown in Fig. 4*B* (lanes 5 and 6), the distribution of pIgA-R was ≈50% in the pellet and ≈50% in the supernatant, indicating that TCVs associate with the target PM in the absence of fusion. The pIgA-R recovered in the pellet is in the 120-kDa form. The fact that only ≈40% (≈50% minus ≈10% for background) of pIgA-R was associated with the target PM in the absence of ATP (lane 6) but ≈60% of TCVs fuse with the target PM in the presence of ATP (lane 2) might be due to a limiting number

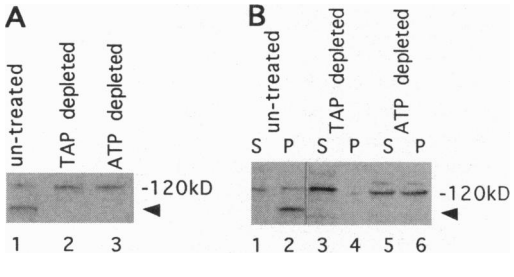

FIG. 4. TAP is required for targeting/binding of TCVs to the target apical PM. (*A*) Reaction mixtures containing untreated cytosol (lane 1), TAP-depleted cytosol (lane 2), or ATP-depleting system (lane 3) were incubated at 37°C for 60 min. Reactions were terminated and pIgA-R was immunoprecipitated and analyzed by SDS/PAGE and fluorography. (*B*) Analogous reaction mixtures were centrifuged and the pellet (P) and supernatant (S) fractions were immunoprecipitated with anti-pIgA-R antibodies. Immunoprecipitates were analyzed by SDS/PAGE and fluorography.

of binding sites on the target PM, suggesting that in the course of a normal reaction, the same PM sites are used for fusion of multiple TCVs.

Molecular Analysis of TAP. The sequence of full-length TAP cDNA (3860 nucleotides) reveals an open reading frame (ORF) of 959 amino acids, 96 nucleotides of 5′ noncoding sequence, and a 3′ untranslated sequence of 884 nucleotides, including a termination codon (nucleotides 2878–80, triple asterisks), a polyadenylylation signal (nucleotides 3721–3726, boldface), and the beginning of the poly(A) tail (nucleotide 2748) (Fig. 5). The sequences of five TAP tryptic peptides obtained by microsequencing are present within the cDNA sequence (boxed residues), indicating that the ORF encodes rat liver TAP.

There are two in-frame ATGs (nucleotides 1–3 and 22–24, marked with single asterisks). The context of the first ATG does not conform to the consensus sequence for eukaryotic translation initiation (17) except for the conserved purine at position −3. The second ATG conforms perfectly to the translation start consensus sequence.

The predicted secondary structure of TAP includes (*i*) an N-terminal region (amino acids 1–650); (*ii*) an internal α-helical region (amino acids 650–930) containing several coiled-coil domains; and (*iii*) a C-terminal acidic region (amino acids 931–959). The N-terminal region contains two proline-rich regions at positions 357–363 and 597–604 (double asterisks). The N-terminal 70-amino acid region is basic, with a calculated pI of 10.22; the C terminus, with a calculated pI of 3.07, is acidic, suggesting that TAP might form a dipole.

The α-helical region contains three domains with strong coiled-coil-forming potential (I, amino acids 650–709; III, amino acids 783–828; IV, amino acids 843–930; shaded letters) as defined by the COILS2 program (18). Another region (≈80% probability of forming a coiled-coil structure) was detected between amino acids 728 and 766 (region II). Helix-breaking prolines and glycines are found flanking each of the putative coiled-coil regions. Electron microscopic analysis (data not shown) indicates that TAP is superficially similar to myosin in that two TAP polypeptides, each composed of a globular (≈9 nm) head and an elongated (≈45 nm) tail, form a homodimer by parallel association of the tails. The coiled-coil region of TAP shows ≈40% similarity and ≈20% identity with other coiled-coil-containing proteins such as myosins, tropomyosins, kinesins, CLIP-170, golgin p160, giantin, and tpr gene product (19–21).

Using the BLAST program we found the strongest homology between TAP and a human cDNA encoding a 99-amino acid ORF with a sequence 92% identical to the TAP sequence (amino acids 605–703 of TAP). A weaker homology was found to Uso1p, a yeast protein involved in ER to Golgi traffic. Uso1p is a hydrophilic protein of 1790 amino acids with a 1010-amino acid coiled-coil region (11). Using BESTFIT (of Genetics Computer Group package) and BLAST programs, we found 42% identity (65% similarity) between amino acids 21–85 of TAP and 18–84 of Uso1p (Fig. 5*B*, boxed residues) and 33% identity (53% similarity) between amino acids 98–259 of TAP and 118–277 of Uso1p. Within this last region there is a stretch of high homology (61% identity, 77% similarity) between amino acids 201–252 of TAP and 220–271 of Uso1p (Fig. 5*B*, boxed amino acids). The C-terminal homology includes the acidic region, which has 85% similarity between amino acids 932–954 of TAP and 1767–1788 of Uso1p (Fig. 5*C*, acidic amino acids in boldface) and the region that immediately precedes it with 43% identity over 37 amino acids (amino acids 898–934) (Fig. 5*C*, boxed amino acids). Interestingly, the sequence immediately preceding the start of the acidic domain, SKLKDLG, is 100% identical in both proteins (double underlined amino acids). The overall homology (28.5% identity between amino acids 1–644 of TAP and 1–760 of Uso1p and 28.65% identity between the coiled-coil domains) of both proteins is relatively low.

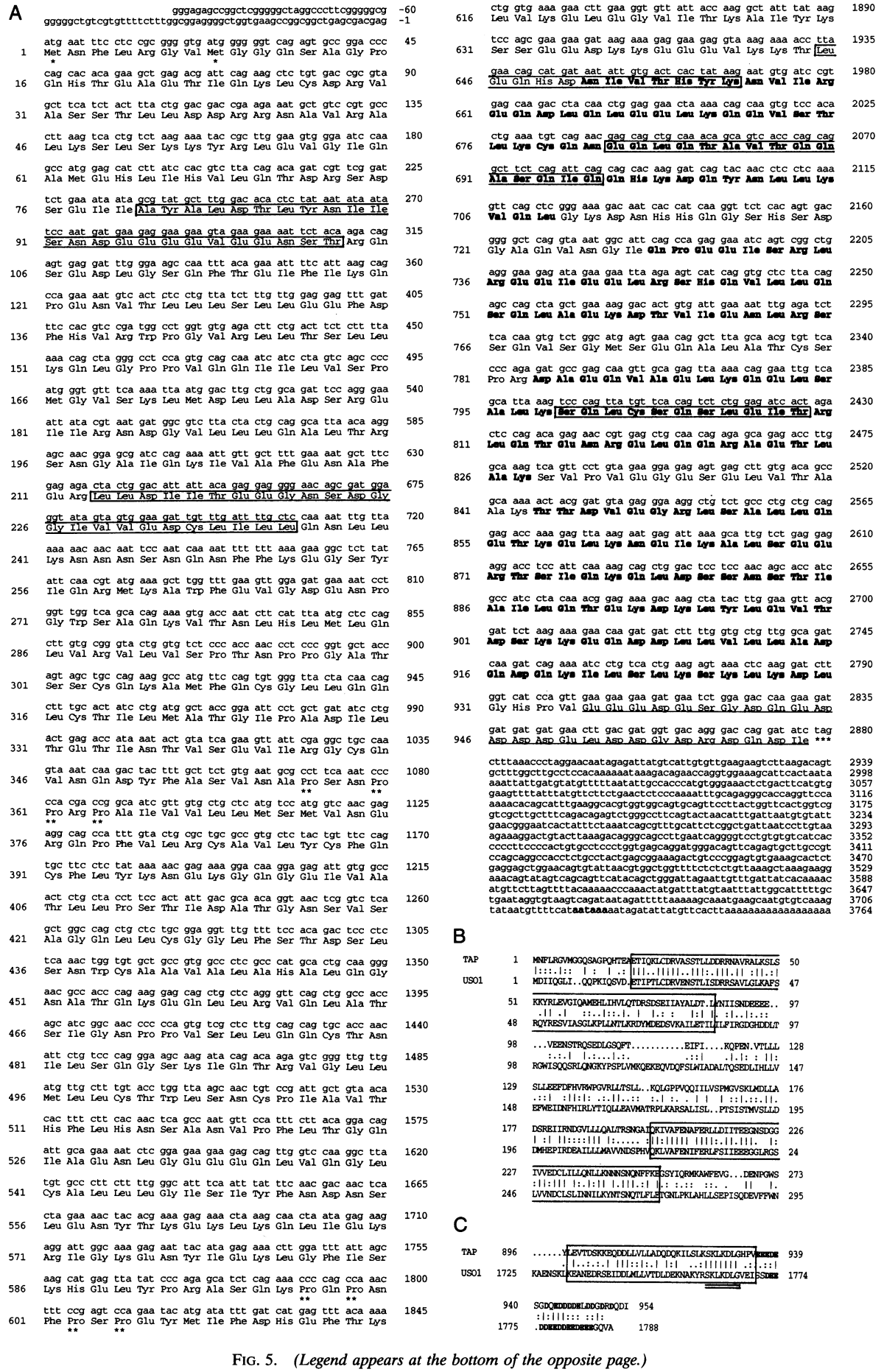

FIG. 5. *(Legend appears at the bottom of the opposite page.)*

The amino acid sequence of TAP was found to be identical to the unpublished sequence of p115 (8), a protein involved in intra-Golgi fusion (9, 10), indicating that TAP/p115 is a general factor operational at multiple membrane fusions.

TAP/p115 and Uso1p are present in the cytoplasm and associated with intracellular membranes (9, 11, 15, 22). TAP was initially identified as a component of TCVs (15), but subsequently we found that TAP is also present on secretory vesicles derived from the TGN and within the Golgi complex (unpublished results). p115 is detected predominantly within the Golgi complex in bovine fibroblasts (9) but its ultrastructural localization is currently unknown. Likewise, the subcellular localization of Uso1p has not been defined but it is likely that Uso1p is present on vesicles operational between the ER and the Golgi complex.

Vesicle targeting and fusion are dependent on reversible and ordered interactions between membrane receptors and soluble cytosolic factors (23, 24). Specifically, proteins present in the vesicles [v-SNAREs; e.g., synaptobrevin (SNARE, SNAP receptor)] would interact with a complementary protein (a t-SNARE; e.g., syntaxin) on the target membrane and this association would form the site for recruitment of the "fusion machinery," a complex that includes NSF and SNAPs (7, 25, 26). Our data showing that ATP-depletion allows the docking of TCVs to the target apical PM supports the hypothesis that the NSF–SNAP complex assembles onto docked vesicles.

How do we superimpose the requirement for TAP/p115 onto the proposed targeting/fusion scheme? Targeting specificities might result from the interactions of v-SNAREs and t-SNAREs. However, vesicle docking prior to targeting might demand the creation of a network of stable interactions between the vesicular and target membranes, facilitating the specific binding of v-SNAREs to t-SNAREs. We propose that TAP/p115 acts as a vesicular "anchor" by interacting with the target membrane and holding the vesicular and target membranes in proximity. This suggestion is based on data from the neuronal field; synaptic vesicles appear normally docked at the target membrane even when v-SNAREs are cleaved by clostridial toxins (and thus unable to form a stable complex), suggesting that SNAREs are not required for association of vesicles with the target membrane and other molecules must perform this function (27). We propose that TAP/p115 may provide such an activity.

FIG. 5 (*On opposite page*). Sequence of TAP and homology alignments. (*A*) cDNA and protein sequence of rat liver TAP. Numbers on right correspond to nucleotide position and those on left correspond to amino acid position. Amino acid sequences of five peptides obtained from tryptic digestion of purified TAP are boxed. The two putative initiator methionines are marked with single asterisks. Stop codon is indicated by triple asterisks. Putative coiled-coil domains are in shaded letters. Proline-rich sequences are marked with double asterisks. Acidic domain is underlined. (*B*) Alignment of TAP and Uso1p N-terminal sequences. Homologous regions are boxed. (*C*) Alignment of TAP and Uso1p C-terminal sequences. Acidic domain is indicated by residues in boldface letters. Homologous region is boxed and a stretch of identity is double underlined.

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