Multiple Residues in the Transmembrane Helix and Connecting Peptide of Mouse Tapasin Stabilize the Transporter Associated with the Antigen-processing TAP2 Subunit*

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The type I endoplasmic reticulum (ER) glycoprotein tapasin (Tpn) is essential for loading of major histocompatibility complex class I (MHC-I) molecules with an optimal spectrum of antigenic peptides and for stable expression of the heterodimeric, polytopic TAP peptide transporter. In a detailed mutational analysis, the transmembrane domain (TMD) and ER-luminal connecting peptide (CP) of mouse Tpn were analyzed for their capacity to stabilize the TAP2 subunit. Replacement of the TMD of Tpn by TMDs from calnexin or the Tpn-related protein, respectively, completely abolished TAP2 stabilization after transfection of Tpn-deficient cells, whereas TMDs derived from distantly related Tpn molecules (chicken and fish) were functional. A detailed mutational analysis of the TMD and adjacent residues in the ER-luminal CP of mouse Tpn was performed to elucidate amino acids that control the stabilization of TAP2. Single amino acid substitutions, including a conserved Lys residue in the center of the putative TMD, did not affect TAP2 expression levels. Mutation of this Lys plus four additional residues, predicted to be neighbors in an assumed α-helical TMD arrangement, abrogated the TAP2-stabilizing capacity of Tpn. In the presence of a wild-type TMD, also the substitution of a highly conserved Glu residue in the CP of Tpn strongly affected TAP2 stabilization. Defective TAP2 stabilization resulted in impaired cell surface expression of MHC-I molecules. This study thus defines a novel, spatially arranged motif in the TMD of Tpn essential for stable expression of the TAP2 protein and a novel protein interaction mode involving an ER-luminal Glu residue close to the membrane.

Major histocompatibility complex class I (MHC-I) molecules bind antigenic peptides for presentation to CD8+ cytotoxic T cells (1). The peptide ligands are usually processed out of proteins by proteasomes in the cytosol and transported into the ER by the ATP-dependent transporter associated with antigen processing (TAP) (2–4). Peptide loading in the ER lumen is facilitated by the ER-resident glycoprotein tapasin (Tpn), which transiently tethers MHC-I heavy chain/β2-microglobulin heterodimers to TAP (5–8). Other components of this peptide-loading complex are the oxido-reductase ERP57, which forms an intermolecular disulfide bridge with Tpn, and the chaperone calreticulin binding to an N-linked glycan in the MHC-I heavy chain (7, 9). In the presence of Tpn, the peptide cargo of most allelic forms of polymorphic MHC-I molecules is optimized, resulting in an altered conformation and improved stability of heavy chain-β2m-microglobulin-peptide complexes on the cell surface (5–6, 9). Tpn binds to exposed loops in the α2 and α3 domains of MHC-I heavy chains through its ER-luminal ectodomain (5, 6).

Of equal importance is the assembly of Tpn with the TAP1 and TAP2 subunits of the heterodimeric peptide transporter. This second function of Tpn is mechanistically independent of the simultaneous binding of MHC-I (10, 11). In human B lymphoblastoid cells, expression of TAP was reduced 3–10-fold in the absence of Tpn (11, 12). In splenocytes from Tpn-deficient mice, TAP expression was found to be reduced more than 100-fold (13), suggesting that there are species-specific and/or cell type-specific differences in the TAP-stabilizing function of Tpn.

Tpn binds to TAP by virtue of its C-terminal portion. The enhancement of TAP steady-state expression levels was completely abolished in the presence of truncated Tpn molecules lacking the transmembrane domain (TMD) and cytoplasmic tail (10, 11). On the other hand, a CD8-Tpn chimera containing the C-terminal part of the connecting peptide (CP), the TMD, and the cytoplasmic tail of Tpn was shown to be functional in terms of TAP stabilization (14). It is, however, only vaguely understood how this region of the Tpn molecule facilitates stabilization of TAP heterodimers. By mutational analysis of human Tpn, a lysine residue (15) and a leucine residue (11) inside the putative TM helix have been implicated in the stabilization of TAP1. An analogous lysine mutation in the TMD of mouse Tpn reduced the association of TAP with Tpn and HC (15).

Since TAP expression was partially retained in the presence of the published Tpn TMD point mutants, we hypothesized that the TAP-stabilizing function of Tpn resides in a larger binding motif. A detailed mutational analysis of murine Tpn revealed that a helically arranged motif of amino acids within the TMD of Tpn essential for stable expression of the TAP2 subunit.
the TMD rather than a single Tpn residue is essential for the stabilization of the TAP2 subunit. Independently, a highly conserved glutamic acid residue in the CP adjacent to the ER interface of the TMD was found to be critical for stable TAP2 expression.

**EXPERIMENTAL PROCEDURES**

Cloning of Tapasin Mutants and TAP1/2-EGFP Constructs—Mouse Tpn (mTpn) cDNA (11) was cloned into the expression vector pcDNA3.1+ (Invitrogen). The naturally occurring splice variant containing an additional Val after mTpn residue 436 was cloned from a BALB/c spleen Marathon-Ready cDNA library (Clontech/BD Biosciences) using the Tpn-specific forward and reverse primers 5′-TCAGAGGCGGCCCCAGGAA-GTT-3′ and 5′-TGAGGGAGAAGAGAAAGAGG-3′, respectively. Following sequencing, the PCR product was subcloned into pcDNA3.1+/mTpn as an XbaI fragment. To generate the ER retrieval signal mutant K463A/K464A of mTpn, the reverse primer 5′-GGCCAAGCTTATTGGAAGCGG-CCTAGTTCCTGGGAATGTGTCAGGCTGGG-3′ was used together with the same forward primer as above and inserted into mTpn cDNA using XbaI and HindIII. Underlined nucleotides refer to sites cut by restriction enzymes. To generate mTpn TMD mutants, an Eco147I site and a Bsp119I restriction site were introduced 5′ of the TMD-encoding sequence at codons 417/419 and 445/447, respectively, using in nested PCRs, the forward primer 5′-ATCGAGGACCAGCCTAGAGGCGG-ACTTCTGGAAATTTGTCAGGCTGGG-3′, reverse primer 5′-GGCCCTTCTCCTGCGA-ACTTCAAGGATGGT-3′, T3 and T7 primers, and a C-terminal XbaI-HindIII mTpn cDNA fragment in pBluescript KS II+ (Stratagene, Amsterdam, The Netherlands) as template. Various single-stranded oligonucleotides containing the desired single or multiple mutations in the TMD of mTpn, a truncated variant of the mTpn TMD, TMD sequences from chicken or (mutated) zebrafish Tpn, or the human Tpn-related protein were synthesized, hybridized with the reverse primer 5′-CGAAACTTCAGGAAAT-3′, filled in using T4 DNA polymerase, phosphorylated with T4 polynucleotide kinase, and a CP/TMD sequence derived from the Tpn-related protein was likewise inserted between Bsp120I and Bsp119I. A CP/TMD sequence containing human calnexin cDNA was kindly provided by W. Hagmann (DKFZ, Heidelberg). The TMD or CP/TMD of human calnexin was cloned in a PCR using the forward primer 5′-GGCTATGCGCCTATGCTGTGGT-AGTCTATATT-3′ or 5′-CTTCTCAGGCGGCCAGCTGAGAGCGCCCGCTGGG-3′, respectively, together with the reverse primer 5′-CTTCTCAGGAAATCTCGAGATGTCGTTTCTTCTTTCCAGAAGGAGGAGGATAC-3′, and the PCR products were digested with Eco471I/Bsp119I or Bsp120I/Bsp119I, respectively, before subcloning.

Murine TAP1 and TAP2 cDNAs have been described (16). The mTAP1 primer pair 5′-TTGATCCGGAAAGCCACTCCTGCTT-3′ and 5′-CTTCTGTCGACGGTACCCAGTCTCGAGGAGGAGGAGGAGGAGGCTCT-3′ and the mTAP2 primer pair 5′-GCCCCGGCCCCTTGTGCAGAACCCA-3′ and 5′-CAGT-TCTAGAGGTACCAGATGCCCTCCTCCAGGCGCTGCTGACCAG-3′ were used in respective PCRs to delete the stop codons and introduce KpnI sites instead. The PCR products were inserted into mTAP1/2 full-length sequences, which were then subcloned into the SacI/KpnI sites (mTAP1Δstop) or KpnI site (mTAP2Δstop), respectively, of the expression vector pEGFP-N1 (Clontech) in frame with the EGFP open reading frame. Oligonucleotides were synthesized by a core facility of the German Cancer Research Center. Restriction enzymes and DNA-modifying enzymes were all from Fermentas (St. Leon-Roth, Germany).

Protein sequence alignments were done using the ClustalV in the program MegAlign (DNASTAR Lasergene, Madison, WI). Transmembrane domains were predicted using TMHMM (available on the World Wide Web at www.cbs.dtu.dk), and helix propensities were predicted using PSipred version 2.4 (available on the World Wide Web at bioinf.cs.ucl.ac.uk/psipred/).

Cell Lines and Transfections—The methylcholanthrene-induced cell line MC4 was derived from the Tpn−/− mouse (17). MC4 cells were kindly provided by N. Garbi (DKFZ, Heidelberg, Germany). Constructs were transiently transfected into MC4 by lipofection. One day before transfection, cells were plated in Dulbecco’s modified Eagle’s medium growth medium (Invitrogen) supplemented with 10% fetal calf serum (Biochrom, Berlin, Germany), 10 mM Hepes (Roche, Karlsruhe, Germany), 0.1% 2-mercaptoethanol (Merck), 2 mM l-glutamine, 1 mM pyruvate, and 5% nonessential amino acids (Invitrogen) and grown to 80–90% confluence at the time of transfection. Transfections with the reagent Lipofectamine 2000 were performed according to the manufacturer (Invitrogen). For Western blots, usually 24 μg of tapasin expression plasmids were used for a 10–cm Petri dish, and for flow cytometry, a total of 4 μg of DNA was used per well of a 6-well plate.

**Antibodies**—Rabbit antiserum recognizing calnexin (SPA-860) and calreticulin (SPA-600) were purchased from Stressgen/Biomol (Hamburg, Germany). The rabbit anti-mTpn N-terminal peptide antiserum, Ra2668 (18), was kindly donated by T. Hansen (Washington University, St. Louis, MO). The rabbit antiserum mTAP2.688, raised against the keyhole limpet hemocyanin-coupled mouse TAP2 peptide 688–702, has been described (13). The biotinylated monoclonal antibody (mAb) Y3.1 recognizes a conformational epitope within the α1–α2 domains of H-2Kb class I heavy chain complexes (19).

**Western Blotting**—To determine protein expression levels by Western blot, cells were lysed for 60 min at 4 °C in TBS (150 mM Tris-HCl, 50 mM NaCl, 5 mM MgCl2, pH 7.4) containing 1% Nonidet P-40 (Fluka, Buchs, Switzerland) and complete protease inhibitor mixture (Roche Applied Science). Samples were separated by 10% SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Millipore, Eschborn, Germany). Membranes were blocked with 1% skim milk powder (Merck) in PBS with 0.1% Tween 20 (Sigma) and incubated subsequently with the primary antibody for 1 h. After three washes with PBS, 0.1% Tween 20, 1% bovine serum albumin, membranes were incubated with anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody (Dianova, Hamburg,
Germany). After development of Western blots with SuperSignal West Dura ECL substrate (Pierce/Perbio Science, Bonn, Germany), protein bands were quantified using the Lumi-Imager System and LumiAnalyst 3.x software (Roche Applied Science).

For endoglycosidase H digestions, the Nonidet P-40 lysate from the ClustalV program of CP, TMD, and cytoplasmic tail sequences of Tpn molecules from mice (Mus musculus, AF043943), rats (Rattus norvegicus, NM_033098), cows (Bos taurus, XM_879476), humans (Homo sapiens, AF009510), dogs (Canis familiaris, XM_538661), chickens (Gallus gallus, AJ004999), zebras (Danio rerio, AF13738), and rainbow trout (Oncorhynchus mykiss, DQ092325). Membrane helices predicted by the program TMHMM version 2.0 are shown in boxes. In fish Tpn sequences, no TMDs were predicted; residues with TMHMM membrane scores of >0.2 are shown in dashed boxes. Helical secondary structures were predicted using PSpred version 2.4. Residues with high helix probabilities (scores 6–9) are shown in italic type. The conserved acidic motif in the CP, the conserved basic residue in the center of the TMD, and the KKXX ER retrieval motif at the C-terminal end are shown in boldface type.

**RESULTS**

**Comparison of Tapasin Sequences from Different Species**—Fig. 1 shows an alignment of CP, TMDs, and cytoplasmic tail sequences of Tpn molecules from mice, rats, cows, humans, dogs, chickens, trout, and zebras, with boxes indicating the TMD as predicted by the TMHMM program. No TMDs were predicted for the two fish Tpn sequences, but residues with TM helix scores of >0.2 are boxed. The secondary structure prediction program PSpred indicated a high helix propensity (score 6–9) for the N-terminal portion, ranging from the N-terminal end of the putative TMDs to a highly conserved basic amino acid or 1 residue beyond, and a lower helix propensity (score <6) for the C-terminal portions of the TMDs. Comparing the mammalian with the nonmammalian Tpn sequences, the C-terminal portion is less conserved in length and sequence homology than the N-terminal portion. In the CP, we noted a conserved cluster of two highly conserved acidic residues (Glu-Asp) located close to the TMD. Except for a KKXX ER retrieval motif (20) at the C-terminal end of all Tpn sequences, there is no apparent homology among cytoplasmic tail sequences from species Tpn/−/− fibroblast line to explore the molecular mechanism of TAP stabilization in the presence of transfected wild-type and mutant Tpn molecules. In a first series of point mutants, we investigated a potential role of the Glu414/Asp415 motif, the KKXX ER retrieval signal, the conserved amino acids Leu433 and Trp435, and the aromatic cluster Tyr439/Trp440 in the C-terminal portion of the TMD of mTpn. By introducing a Val residue after Leu436, we mimicked a naturally occurring mTpn splice variant (14).

Point mutants of mTpn were transiently transfected into Tpn-deficient MC4 fibroblasts. After 48 h, TAP2 stabilization was analyzed by quantification of chemoluminescence signals in Western blots of total cell lysates. Following transfection of wild-type (WT) mTpn, mTpn[L433F], mTpn[W435L], mTpn[V436A], mTpn[Y439A], and mTpn[Y439A/W440L], similar levels of mTAP2 were detected (Fig. 2A and Table 1), suggesting that the properties of these amino acids were not critical for the TAP2-stabilizing function of the TMD. Also, the mTpn[K463A/K464A] mutant was able to substitute WT mTpn, suggesting that ER retrieval/retention is not essential for the TAP2-stabilizing function of Tpn. In striking contrast, the mTpn[E414K/D415N] mutant almost completely lost the capacity to enhance mTAP2 expression, although this Tpn mutant was expressed at normal levels in transiently transfected H-2HA mutant fibroblasts if cotransfected with the corresponding untagged subunit as indicated by enhanced MHC-I surface expression of EGFP-positive cells (data not shown).
To assess the functional relevance of TAP2 stabilization by the various Tpn TMD mutants, we analyzed the peptidetransport-dependent induction of MHC-I molecules in MC4 cells. EGFP was co-transfected together with Tpn mutants, and after 48 h, the Kb surface expression of EGFP-positive cells was analyzed by flow cytometry. As compared with cells transfected with the empty pcDNA3.1 vector, we observed a 3–4-fold Kb induction in the presence of wild-type mTpn and the above mentioned mTpn point mutants except mTpn[E414K/D415N] (Table 1 and Fig. 4D).

The substitutions Y439A/W440L of the aromatic cluster at the C-terminal end of the TMD of mTpn reproducibly caused a slower migration of the mutant Tpn molecule in SDS-PAGE (Fig. 2, A and D). This was not the case for mTpn[Y439A]. After treatment of lysates with endoglycosidase H, mTpn[Y439A/W440L], mTpn[Y439A], and WT mTpn migrated similarly, suggesting that the apparent shift in molecular weight was due to overglycosylation of the former mutant (Fig. 2D). Sequencing of the mTpn[Y439A/W440L] plasmid revealed, however, no inadvertent mutation in the ectodomain, which normally harbors a single N-glycan consensus sequence.

The Connecting Peptide of Tpn Is Not Sufficient to Stabilize TAP2—Since the acidic cluster in the CP appeared to be essential for the TAP-stabilizing function of Tpn, we next wished to determine whether the residues Glu414/Asp415 alone would be sufficient to mediate stabilization of TAP2. To this end, the TMD of mTpn was replaced by heterologous TMDs derived from the ER-resident protein calnexin (Cnx) and the surface-expressed tapasin-related protein (TpnR), respectively, leaving the CP of mTpn intact (Fig. 3A). In two other chimeras, the mTpn TMD and the mTpn CP residues SI EDG were replaced by the respective sequences from Cnx and TpnR. The chimeras mTpn[Cnx-CP/TMD] and mTpn[Cnx-TMD] retained only a minor capacity to stabilize TAP2 above the levels of vector-transfected or untransfected MC4 cells. In conclu-
sion, the mTpn CP alone cannot render the heterologous TMDs from Cnx and TpnR competent to enhance TAP2 expression. Apparently, the acidic cluster is not sufficient to facilitate TAP2 stabilization. The lack of Kb induction in the presence of these TMD chimeras mirrored the low TAP2 levels (Fig. 3D).

In contrast to the TMDs from Cnx and TpnR, the TMD of chicken Tpn, which is predicted to be 4 residues shorter in the C-terminal portion than mTpn (Fig. 1), was fully functional when inserted between the CP and cytoplasmic domain of mTpn (Fig. 3C). Chimeric mTpn containing the TMD of zebrafish Tpn (zTpn) also induced TAP2 expression, however, to only about 40–50% of the WT mTpn level (Fig. 3B and C). Since chicken Tpn shows a higher degree of homology with mTpn in the N-terminal portion of the TMDs than zTpn (Fig. 1), these findings suggest that sequence information contained within the N-terminal portion of Tpn is crucial for TAP2 induction. By substituting residues Val410, Ala411, and Leu414 within the zTpn TMD by the hydrophobic and more bulky residues Phe, Leu, and Phe from mTpn, respectively, we rendered the zTpn TMD chimera fully functional in terms of TAP2 enhancement (Fig. 3C). In contrast, extending the zTpn TMD at the N-terminal end by the naturally occurring amino acids Met 407 and Ala408 further reduced the capacity of this Tpn chimera to enhance TAP2 expression (Fig. 3C).

To further assess the relevance of the N-terminal region of the TMD, we deleted the C-terminal portion (VLGWLAAYW) in the TMD of WT mTpn (Fig. 3A). Tpn with truncated TMD could only be weakly expressed in transiently transfected MC4 cells, which is probably due to inefficient membrane insertion of this minimal transmembrane helix (15 amino acids) (Fig. 3B). Nevertheless, TAP2 induction was still observed at about 70% of the WT mTpn level, confirm-

### TABLE 1
| Sequence | Mutants | %TAP2 expression | %Kb expression |
|----------|---------|------------------|----------------|
| POINT MUTATIONS IN THE C-TERMINAL REGION OF THE TMD OR IN THE KXXX MOTIF |   |   |   |
| ...SIEDEGLFLAFDLLGLKVLGLAAAYTI...KKSQ* | wild-type mouse Tpn | 100 | 100 |
| | no Tpn | 0-17 | 22-26 |
| | K431E | 112 | 94 |
| | K431D | 119 | 93 |
| | K431Q | 117 | 95 |
| | K431G | 133 | 88 |
| | K431L | 140 | 106 |
| | K431A | 132 | 106 |
| | L433F | 98 | n.a. |
| | W435L | 102 | n.a. |
| | V436a | 110 | n.a. |
| | Y439A | 126 | 93 |
| | Y439A/W440L | 114 | n.a. |
| | K463A/K464A | 88 | n.a. |
| POINT MUTATIONS IN THE N-TERMINAL REGION OF THE TMD | F420V/F424A | 141 | 105 |
| | F424A | 157 | 124 |
| | G428I | 100 | 89 |
| POINT MUTATIONS IN THE CONNECTING PEPTIDE | E414K/D415N | 16 | 44 |
| | E414S | 25 | 36 |
| | D415S | 110 | 97 |
| | E414S/D415S | 45 | 77 |
| MULTIPLE MUTATIONS | F420V/F424A/G428I/K431A | 35 | 88 |
| | G428I/K431A/W435L | 59 | 84 |
| | F424A/G428I/K431A/W435L | 17 | 41 |
| | E414S/F420V/F424A/G428I/K431A | 13 | 38 |
| | E414S/F424A/G428I/K431A/W435L | 16 | 35 |

* Predicted TMD shown in italics. Mouse Tpn residues subjected to mutation are underlined. Numbering according to mTpn open reading frame.
* TAP2 expression determined by Western blot; band intensities are normalized to TAP2 expression in the presence of wild-type mTpn.
* Kb expression determined by cytofluorometry; mean fluorescence values are normalized to Kb expression in the presence of wild-type mTpn.
* Range of several experiments.
* n.a., not analyzed.
ing the importance of the N-terminal portion. In accordance with the diminished TAP2 stabilization, mTpn containing the zTpn TMD induced only reduced Kb cell surface expression levels in MC4 cells, whereas no induction was observed in the presence of mTpn with the N-terminally extended zTpn TMD (Fig. 3D).

FIGURE 3. Loss of TAP2 stabilization in the presence of mTpn chimeras containing TMDs from unrelated proteins. A, substitution of the TMD of mTpn by TMD sequences from chicken Tpn, zebrafish Tpn, the Tpn-related protein (TpnR) (AK002056), or calnexin (M98458). Nonmurine amino acids are underlined. Chimeras with TpnR or calnexin sequences harbor either the heterologous CP and TMD sequences or the mTpn CP and the heterologous TMD. Zebrafish Tpn TMD chimeras containing either three substitutions analogous to the mTpn sequence (*italic type*) or two additional zebrafish Tpn-derived amino acids at the N-terminal end (*italic type*), respectively, and a truncated version of the mTpn TMD are also shown. B and C, Western blot analysis of mouse TAP2 expression levels in MC4 cells transfected for 48 h with the indicated mTpn mutants, wild-type mTpn, empty pcDNA3.1 expression vector, or no DNA for control. The intensity of mTAP2 bands normalized to wild-type mTpn was quantified. Tpn expression was monitored in the same Western blot using anti-Tpn antiserum. Blots stained with anti-Cnx or anti-calreticulin (Crt) antisera served as loading controls. D, induction of Kb cell surface expression following transfection of Tpn mutants. Tpn-deficient MC4 cells were transfected for 48 h with pEGFP-N1 plus WT mTpn, the indicated mTpn chimeras, or empty pcDNA3.1+ vector for control. Kb expression of EGFP+ cells is analyzed by flow cytometry using mAb Y3. In each image, one representative of four or five experiments with similar results is shown.
Mutations in the Connecting Peptide—Next we inspected whether single substitutions of Glu^{414} or Asp^{415} would affect the stabilization of TAP2 similar to the double mutant E414K/D415N (Figs. 2A and 4A). These amino acids were individually or concomitantly replaced by Ser. The mTpn[D415S] mutant showed normal TAP2 induction, whereas the E414S mutation resulted in a complete loss of function (Fig. 4B and Table 1). This suggests that residue Glu^{414}, but not Asp^{415}, is critical for the stabilization of TAP2. The sequence context of amino acid 414 appears to matter, however, since the destabilizing effect of the E414S mutation was less pronounced if also Asp^{415} was mutated to Ser (Fig. 4B). Combination of the CP mutant E414S with mutations of the spatially arranged motif FXXFXXXXGXXK or FXXXGXXXXXXXXX (see Fig. 5), respectively, also resulted in a complete loss of TAP2 induction (Fig. 4C). In the presence of those CP mutants that were associated with briskly reduced TAP2 expression levels, Kb expression was clearly below that of WT mTpn in the presence of the control transfectant (Fig. 4D).

A Spatially Arranged Motif in the TMD of mTpn Is Involved in TAP2 Stabilization—Within the C-terminal half of the TMD, substitution of Lys^{431} by various amino acids and the replacement W435L alone did not affect the function of mTpn. Assuming a helical conformation of the TMD, the aromatic residues Phe^{420}, Phe^{424}, and Trp^{435} as well as residues Gly^{428} and Lys^{431} are predicted to locate to the same flank of the helix, while being distributed along the membrane-spanning vertical axis (Fig. 5A). We hypothesized that the exact composition and spatial arrangement of these 5 residues were critical for the function of Tpn. Substitution of G428 by the more bulky amino acid isoleucine and substitutions of Phe^{424} and Phe^{420}/Phe^{424} by the less bulky aliphatic amino acids Val/Ala, however, did not detectably diminish the capacity of the mutants to stabilize mTAP2 (Fig. 5, B and C). In the presence of the triple/quadruple point mutants mTpn[G428I/K431A/W435L], mTpn[F420V/F424A/G428I/K431A], and mTpn[F424A/G428I/K431A/W435L], TAP2 stabilization was reduced to 59, 35, and 17%, respectively, of WT mTpn levels (Fig. 5, B and C). This finding suggests that Lys^{431} and its helical neighbors are required for a fully functional TMD. In agreement with low TAP2 levels, induction of Kb cell surface expression was clearly below that of WT mTpn in the presence of the TMD mutant mTpn[F424A/G428I/K431A/W435L] (Fig. 5D). Thus, the peptide transport capacity appears to become a limiting factor in the presence of low but not intermediate TAP2 steady-state levels.

DISCUSSION

The ER-resident glycoprotein tapasin is an accessory molecule assisting the loading of MHC class I molecules with antigenic peptides. Tpn not only bridges MHC-I molecules to the peptide transporter-associated loading complex and thereby optimizes the peptide cargo and stability of variousalloforms of polymorphic MHC-I molecules (5–7); Tpn is also essential for a stable expression of the peptide transporter itself and is thus an indispensable component of the MHC-I antigen-processing pathway (10–12).

Previous investigations suggested that a C-terminal region containing the TM domain and adjacent connecting peptide and cytoplasmic tail sequences exerted the TAP-stabilizing function of Tpn (5, 10–12, 14, 15). By excising the TMD of Tpn and replacing it by the heterologous TMDs derived from calnexin and the tapasin-related protein, we have shown here for the first time that the TMD itself is essential as a TAP stabilizer, since a complete loss of function was entailed by this replacement (Fig. 3). Formally, the TAP-stabilizing function of Tpn has only been demonstrated for human (5, 10–12, 15) and murine cells (14, 15, 21, 22). Furthermore, mTpn was demonstrated to efficiently stabilize hTAP (11). Considering our herein reported findings that the TMDs from the distantly related chicken and
zebrafish Tpn molecules are able to stabilize mTAP2 (Fig. 3), it seems likely that the TAP-stabilizing function of Tpn evolved together with the TAP peptide transporter, which first appears in vertebrates.

The comparison of Tpn sequences from eight species revealed that the TMD can be subdivided into an N-terminal region of 15–16 residues ending with the conserved basic amino acid Lys(Arg), which is predicted to possess a high $\alpha$-helix propensity, and a C-terminal region of 4–8 residues of lower predicted helix propensity. This C-terminal region is poorly conserved between mammalian and nonmammalian Tpn sequences. Our results indicate that the N-terminal TMD region plays a major role in the TAP-stabilizing function for the following reasons: (i) deletion of the C-terminal region in the TMD of mTpn did

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**FIGURE 5.** Mutation of helically arranged mTpn TMD residues results in loss of TAP2 stabilization. A, helical wheel projection of the putative TMD of mTpn. The circled residues Phe$^{420}$, Phe$^{424}$, Gly$^{428}$, Lys$^{431}$, and Trp$^{435}$ are vicinal on the same side of an assumed transmembrane $\alpha$-helix. A putative leucine heptad (Leu$^{419}$/Leu$^{429}$/Leu$^{433}$) motif is indicated by a curve. Residues Ile$^{417}$/Gly$^{418}$ at the N-terminal end and Ala$^{437}$/Ala$^{438}$/Tyr$^{439}$ at the C-terminal end of the predicted TMD are not included in the wheel. B and C, Western blot analysis using rabbit anti-mTAP2 antibodies in MC4 cells transfected for 48 h with the indicated mTpn mutants, wild-type mTpn, or pcDNA3.1+ vector for control. The intensity of TAP2 bands was quantified and normalized to wild-type mTpn. Tpn expression is monitored in the same Western blot. Tpn expression was analyzed on the same blot using anti-Tpn antiserum. Blots stained with Cnx-specific antiserum serve as loading controls. D, induction of Kb cell surface expression following transfection of the indicated Tpn mutants plus EGFP for 48 h. Kb expression of EGFP$^{+}$ cells was analyzed by flow cytometry using mAb Y3. In each image, one representative of four or five experiments with similar results is shown.
not abolish TAP2 stabilization, although the truncation mutant possessing only a minimal 14-residue TMD was ineffectively expressed; and (ii) the TMD of chicken Tpn, which contains the C-terminal TMD sequence WLYP instead of VLGWLAAAY in mTpn, was fully functional in an mTpn chimera. Nevertheless, the amino acid Trp\(^{345}\) in mTpn, which is conserved except for chicken Tpn, seems to be involved in TAP2 stabilization and could be part of a helical interaction site. This is concluded from the finding that the combined mutation of residues F\(^{424}\)XXG\(^{428}\)XXK\(^{431}\)XXW\(^{435}\) had a stronger negative impact on TAP2 stabilization and MHC-I induction than the mutation of F\(^{420}\)XXXT\(^{426}\)XXXG\(^{428}\)XXK\(^{431}\) (Fig. 5). In the sequence context of the chicken Tpn TMD, proline might functionally substitute for Trp\(^{345}\) or, at least, does not exert a destabilizing effect. The aromatic residues Tyr\(^{349}\) and Trp\(^{340}\) that have a high probability of occurring at membrane interfaces (23) could be replaced by aliphatic residues without any loss of function. Thus, these bulky residues do not appear to play a significant role in the Tpn/TAP interaction. Consistent with this notion, the inclusion of amino acid Val\(^{436a}\) did not alter the TAP2-stabilizing function of mTpn. In the presence of the additional residue Val\(^{436a}\), Tyr\(^{439}\) and Trp\(^{440}\) would be shifted by 100° within an α-helix. If the spatial position of Tyr\(^{349}\)/Trp\(^{340}\) was of importance for TAP2 stabilization, the splice variant Val\(^{436a}\) should have affected this function.

Single amino acid substitutions within the N-terminal region of the mTpn TMD (F424A, G428I, K431A/L/G/Q/E/D, and L433F) did not entail any detectable loss of mTAP2 stabilization and the TAP-dependent induction of surface Kb molecules (Fig. 2). This finding was unexpected, since mutation of the human analogs of mTpn residues Lys\(^{431}\) and Leu\(^{433}\) has been reported to impair steady-state expression levels of the human TAP1 subunit (11, 15). Furthermore, the mutation K431A in mTpn was found to affect the assembly of Tpn as human TAP1 subunit (11, 15). Consequently, the mutants W435L, F420V/F424A/G428I/K431A, and F424A/G428I/K431A/W435L resulted in clear reductions of TAP2 steady-state expression levels, whereas the Tpn mutants themselves were expressed normally (Fig. 5). This reduction was most pronounced in the latter 4-fold mutant. We conclude that a spatially arranged sequence motif comprising 4–5 defined residues located on the same flank of the TMD helix is involved in the interaction of Tpn with the TAP2 subunit. The proposed (F)XXXFXXYGXXXWW motif would be different from the GXXXXG-based TM oligomerization motifs (26–28) and the QXXS motif (29) described so far. Corresponding interaction sites within the TMDs of TAP2 and TAP1 still need to be determined, however, before final conclusions about the molecular mechanism of the Tpn/TAP2/TAP1 assembly can be drawn. Recent results suggest that the N-terminal first TMD of the polytopic TAP2 and TAP1 proteins, respectively, are involved in Tpn binding (30). If these TMDs, whose exact boundaries are difficult to predict, can be confirmed to directly associate with the TMD of Tpn, it should be a feasible task to identify Tpn-binding amino acids or sequence motifs within TAP2 and TAP1.

Independent of mutations within the TMD of Tpn, mutation of the highly conserved Glu\(^{414}\)/Asp\(^{415}\) cluster flanking the TMD from the ER-luminal side disrupted the TAP2-stabilizing function of mTpn. Interestingly, only the acidic charge at position 414 seems to be of functional importance, since the single mutation D415S did not affect TAP2 expression. Impaired hTAP2 expression in the presence of the hTpn mutants E411K/D412N and E411S could be confirmed in Tpn-transfected human .220 cells. In all known Tpn sequences, the conserved Glu is spaced by 16 residues from the conserved Lys within the TMD. This spacing seems to be important for the TAP-stabilizing function, since introduction of an additional Met/Ala at the N-terminal end of the TMD in the zebrafish/mouse chimera resulted in a strongly reduced TAP2 stabilization (Fig. 3).

The ER-resident chaperone Cnx participates in a TAP-associated precursor complex preceding the formation of the final peptide-loading complex, including MHC-I (11, 31, 32). Cnx was recruited to TAP in the presence of a truncated Tpn

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3 M. Papadopoulos and F. Momburg, unpublished observation.
mutant lacking almost the entire ectodomain (31). Since Cnx contains an Arg residue in the CP close to the N-terminal border of its TMD (see Fig. 3A), this Arg might interact with the Glu/Asp motif of Tpn. We presently study whether the Tpn/Cnx interaction is disturbed by mTpn CP mutations.

In fluorescence energy transfer experiments, yellow fluorescent protein-tagged Tpn molecules have been found to be mostly present in clusters that rapidly diffuse within the ER membrane (33). We speculate that the acidic CP cluster in the vicinity of polar head groups of (phospho)lipids in the ER membrane might regulate the dynamic entry and exit of Tpn molecules into and from more slowly moving TAP-containing clusters (33, 34). It will be interesting to investigate the clustering and ER membrane mobility of, for example, the mTpn[E414K/D415N] mutant.

It is interesting to note that mutation of the C-terminal ER retrieval motif KKSQ to AASQ did not affect the capacity of mTpn to stabilize TAP2. Whereas in one study, no evidence was found for a significant ER exit and cycling of yellow fluorescent protein-tagged hTpn molecules in HeLa cells (33), other reports suggested that the KKXX motif controls the COPI vesicle-mediated, retrograde transport of hTpn from the cis-Golgi to the ER (35, 36). Upon mutation of the dilysine motif, an increased proportion of hTpn located to the Golgi (36). On the other hand, TAP was found associated with COPI vesicles (35), increased proportion of hTpn located to the Golgi (36). It is interesting to note that mutation of the C-terminal ER retrieval motif KKSQ to AASQ did not affect the capacity of mTpn to stabilize TAP2. Whereas in one study, no evidence was found for a significant ER exit and cycling of yellow fluorescent protein-tagged hTpn molecules in HeLa cells (33), other reports suggested that the KKXX motif controls the COPI vesicle-mediated, retrograde transport of hTpn from the cis-Golgi to the ER (35, 36). Upon mutation of the dilysine motif, an increased proportion of hTpn located to the Golgi (36). On the other hand, TAP was found associated with COPI vesicles (35), and it was detected in cis-Golgi vesicles by immune electron microscopy (37). Since the mutant mTpn[K462A/K463A] was fully functional in terms of TAP2 stabilization, it is conceivable that Tpn acts on TAP in the ER proper and also within the recycling pathway.

Taken together, Tpn plays a unique role as a dedicated chaperone for the peptide transporter TAP, which requires Tpn for stable assembly. In the present study, we have provided insight into the molecular mechanism of the Tpn/TAP interaction and defined two independent elements critical for this interaction. The spatially arranged F/F/G/K/W cluster in the transmembrane domain of mouse Tpn and the E/D cluster in the connecting peptide represent novel motifs for intermolecular interaction.

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