Integrity of all four transmembrane domains of the tetraspanin uroplakin Ib is required for its exit from the ER

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Summary

The surface of the mammalian urinary bladder is covered by a crystalline, asymmetric unit membrane (AUM) structure that contains the four major uroplakins (UPs): Ia, Ib, II and IIIa. UPIa and UPIb belong to the family of tetraspanins. Although UPIa and UPIb are structurally conserved, only UPIb could exit from the endoplasmic reticulum (ER) and reach the cell surface when expressed alone in 293T cells. Modifications of the large extracellular loop of UPIb, such as mutation of the N-glycosylation site or the cysteines involved in the formation of three disulfide bridges, or exchanging the large luminal loop of UPIb with that of UPIa did not affect the ability of UPIb to reach the cell surface. However, modifications of any of the four transmembrane domains of UPIb led to ER retention, suggesting that the proper formation of helical bundles consisting of the tetraspanin transmembrane domains is a prerequisite for UPIb to exit from the ER. Results of sedimentation analysis suggested that aggregate formation is a mechanism for ER retention.

Key words: Uroplakins, Tetraspanins, Endoplasmic reticulum, Membrane proteins

Introduction

The mammalian urinary bladder is lined by urothelium, a stratified epithelium that protects the underlying tissues from the potentially harmful effects of the urine content. The urothelium accomplishes this by forming a superficial layer of highly differentiated umbrella cells that elaborate a specialized, apically located asymmetric unit membrane (AUM). This unique membrane structure is comprised mainly of four integral membrane proteins, the uroplakins (UPs) Ia, Ib, II and IIIa (Wu et al., 1990; Wu and Sun, 1993; Lin et al., 1994; Wu et al., 1994; Yu et al., 1994; Deng et al., 2002). The UPs undergo an assembly process that begins in the ER where they form specific heterodimers (Wu et al., 1995; Liang et al., 2001; Tu et al., 2002). In a post-Golgi compartment they form increasingly larger crystalline arrays (Hicks, 1965; Porter et al., 1967; Staehelin et al., 1972; Wu et al., 1990; Kachar et al., 1999), which ultimately cover almost the entire surface of the mature fusiform vesicles. These organelles migrate towards the apical cell periphery where they can fuse with the apical plasma membrane (Hicks, 1965; Porter et al., 1967; Kachar et al., 1999; Hu et al., 2000).

UPIa and UPIb belong to the tetraspanin family that includes CD9, CD63, CD81, CD82 and CD151 (Hemler, 2003). Tetraspanins have four transmembrane (TM) domains that contain several highly conserved and charged amino acids, a small extracellular domain interconnecting TM1 and TM2 and a large one interconnecting TM3 and TM4. As is the case for many other tetraspanins, the large loop of UPIa and UPIb carries an N-linked oligosaccharide (Yu et al., 1994; Hemler, 2003). Sequence comparisons revealed that the tetraspanins can be divided into groups with two, three or four disulfide bridges in their extracellular loops (Seigneuret et al., 2001). UPIa and UPIb belong to a group with three disulfide bridges. We have previously investigated the early stages of uroplakin assembly by expressing the four major uroplakins individually or in pairs in 293T cells. Although UPIa is transported to the plasma membrane only after heterodimerization with UPII, UPIb is unique in that it can exit from the ER and move to the plasma membrane even when expressed by itself (Tu et al., 2002). The fact that UPIb can exit from the ER without forming a heterodimeric complex is also consistent with the previous finding that UPIb can be detected at the surface of urothelial cells of UPIIIa-knockout mice, although the immunohistochemical evidence suggests that delivery of this tetraspanin may occur predominantly to the basolateral surface of the plasma membrane (Hu et al., 2000). On the other hand, in UPII-knockout mice UPIa remains trapped in the ER (Kong et al., 2004).

Here, we have investigated possible mechanisms that allow UPIb to exit from the ER, but trap UPIa in this compartment. By generating UPIa/UPIb chimeras through a systematic exchange of domains between the two tetraspanins, and by constructing deletion- and site-specific UPIb mutations, we demonstrate that the four TM domains of UPIb contain sequence and structural information that may affect specific interactions among the TM domains that are crucial for the transport of UPIb from the ER to the Golgi complex.

Results

Swapping domains of UPIa and UPIb

We have previously shown that when UPIb is expressed alone in 293T cells, it can reach the cell surface, whereas the closely related UPIa remains trapped in the ER (Tu et al., 2002). Possible mechanisms that prevent UPIa from leaving the ER include: (1) misfolding of luminal domains that are recognized by chaperones that form a part of the ER quality control system (Ellgaard and Helenius, 2003); (2) improper assembly of the TM domains (Cannon and Cresswell, 2001; Schamel et al., 2003; Swanton et al., 2003; Krebs et al., 2004; Kota and Ljungdahl, 2005); (3) lack of an ER exit signal (Barlowe, 2003); or (4) formation of large aggregates (Ellgaard and Helenius, 2003). We have performed experiments to define the roles of different domains of UPIb that allow it to exit from the ER, and to gain insight into the mechanisms involved in this process.

Since UPIa and UPIb are structurally related (Min et al., 2003), we generated UPIa/UPIb chimeras and tested their ability to exit from the ER. As members of the tetraspanin family, UPIa and UPIb share characteristic features including a small and a large extracellular loop and four TM domains. A comparison of the amino acid sequences of bovine UPIa and UPIb revealed a striking degree of sequence identity between their corresponding TM domains, i.e. 39% for the first, 26% for the second, 64% for the third and 54% for the fourth one (Fig. 1A), suggesting that these TM domains may function beyond simply providing membrane anchorage. Of special interest were the potentially negatively charged glutamic acid residues in TM domains 1 and 3 (Glu37 and 107 in UPIa, Glu32 and 102 in UPIb), of which Glu107 in TM domain 3 is highly conserved in the tetraspanin family (Hemler, 2003). Schematic representations of UPIa and UPIb shown in Fig. 1B provide information on the proposed boundaries of the TM domains (Yu et al., 1994), and on the definition of subdomains 1 to 9.

We constructed 18 UPIa/UPIb chimeras in which each of the nine domains of the two tetraspanins was systematically swapped (Fig. 1C). Western blot analyses established that all chimeras, even those that did not show surface staining in non-permeabilized cells (see below), were expressed at similar protein levels (Fig. 1D). Although mature AUM plaques contained predominantly the N-glycosylated forms of UPIa and UPIb (asterisks in Fig. 1D), it should be pointed out that the N-linked oligosaccharide of both UPIa and UPIb remain endoglycosidase-H (Endo H) sensitive even when they transverse the Golgi complex and are expressed at the cell surface (Tu et al., 2002). When the two uroplakins were expressed in 293T cells, two bands can be detected by western blot, one corresponding to the N-glycosylated protein and the other band of higher mobility corresponding to the nonglycosylated form (Fig. 1D). As expected, mutation of the asparagine residue, which normally represents the N-glycosylation site of UPIb, to arginine (UPIbm, Fig. 1D), abolished N-glycosylation completely. That N-glycosylation is not required for transport to the plasma membrane is demonstrated by the fact that the nonglycosylated UPIbm protein was expressed at the surface (Fig. 2A), whereas the completely N-glycosylated UPIb/UPIa4 protein was retained in the ER (Fig. 5A). N-glycosylation is apparently an intrinsic property of the individual chimeric protein and is not related to protein transport to the cell surface. All UPIb mutants that are not expressed at the cell surface show the same ER-labeling pattern demonstrated for UPIa and UPIb (Fig. 2C). Colocalization of these uroplakin-related constructs with the ER marker ribophorin-I-GFP was observed.

The two extracellular loops of UPIb are not involved in mediating exit from the ER

Since all tetraspanins share a common, overall structure, we tested the possible involvement of the extracellular loops in the exit of UPIb from the ER. We found that chimeras UPIb/a3 and UPIb/a7 – UPIb with its small or large loop replaced by the corresponding one of UPIa – were still expressed at the cell surface (Fig. 2A, Fig. 5A). This finding demonstrated that the extracellular loops of UPIa do not contain any specific ER-retention information and are unlikely to be responsible for the retention of UPIa in the ER. Furthermore, mutation of the N-glycosylation site, resulting in a non-glycosylated form of UPIb (UPIbm, Fig. 1D), did not abolish the ability of UPIb to exit from the ER (Fig. 2A). We also found that none of the UPIa/UPIb chimeras in which all the subdomains of UPIa were systematically replaced one at a time by those of UPIb could exit from the ER (data not shown), indicating that more than one domain contributed to the ER retention of UPIa.

Formation of disulfide bridges is an essential step for the proper folding of most membrane proteins (Ellgaard and Helenius, 2003). Tetraspanins have disulfide bridges located in highly conserved positions of the large loop (Seigneuret et al., 2001; Hemler, 2003), and it has been suggested that in UPIb, Cys159, Cys160 and Cys191 form disulfide bridges with Cys218, Cys190 and Cys205, respectively (Seigneuret et al., 2001). To investigate whether disruption of these disulfide bonds causes misfolding of the large loop that would result in the retention of the mutated forms of UPIb in the ER, we replaced Cys159, Cys160 and Cys191 with Ala (UPIbCA; Fig. 1C) and expressed this mutant in 293T cells. Surprisingly, this mutant was still expressed at the cell surface (Fig. 2A), and, compared with native UPIb, no significant increase in ER accumulation was observed in permeabilized cells (Fig. 2B). It appears, therefore, that the disulfide bridges are either not needed for the proper folding of the large loop, or the resulting misfolding does not cause persistent binding of chaperones that would interfere with the exit of this UPIb mutant from the ER.

A tyrosine residue in the cytoplasmic tail is essential for exit of UPIb from the ER, but is not part of an ER-exit signal

Replacement of the short C-terminal cytoplasmic tail of UPIb with the corresponding one from UPIa resulted in the complete retention of the UPIb/a9 chimera in the ER. Although permeabilized cells confirmed the expression of the chimera UPIb/a9 in the ER, immunostaining of intact cells showed no surface staining (Fig. 3A). Since Tyr is frequently found in ER exit signals (Barlowe, 2003), we performed an Ala scan on the ten C-terminal amino acids of UPIb, including a part of the fourth TM domain (Fig. 3B). We found that replacement of Tyr254, located seven amino acids from the C-terminus, with Ala (UPIbA7; Fig. 3C), resulted in the ER retention of this mutated form of UPIb. Interestingly, when this Tyr was replaced with Phe (UPIbF7; Fig. 3B), the mutant was expressed at the cell surface (Fig. 3C). It appears, therefore,

A

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                         TM1                                                    TM2
bUPIa  MASAAAATTEKGSPVVVGLLVMGNIIILLSGLALFAETVWVTADQYRIYP LMGVSGKDDVFAGAWIAIFCGFSFFVVASFGVGAALCRRR
bUPIb  MAKDDSTVR-----CFQGLLIFGNVIIGMCSIALMAECIFFVSDQNSLYP LLEATNNDDIYAAAWIGMSVGICLFCLSVLGIVGIMKSNR
       **    ..     . ***::**:** :..:**:** ::..:**  :** *: .:.:**::*.***.:  *:.:* :: :*: . :  .*
            TM3
bUPIa  SMILTYLILMLIIYIFECASCITSYTHRDYMVSNPSLITKQMLTFYSADS-----NQGRE --LTRLWDRIMIEQECCGTSGPMDWVNFTS
bUPIb  KILLVYFILMFIVYAFEVASCITAATQRDFFT--PNLFLKQMLERYQNNSPPNNDDQWKN NGVTKTWDRLMLQDNCCGVNGPSDWQKYTS
       .::*.*:***:*:* ** *****: *:**::.  *.*: ****  *. :*      :* ::  :*: ***:*::::***..** ** ::**
                                                                                    TM4
bUPIa  AFRATTPEVVFPWPPLCCRRTGNFIPVNEEGCRLGHLDYLFTKGCFEHIGHAIDSYTWGISWFGFAILMW TLPVMLIAMYFYTTL
bUPIb  AFRTENSDADYPWPRQCCVMNSLKEPLNLDACKLGVPGYYHSHGCYELISGPMNRHAWGVAWFGFAILCW TFWVLLGTMFYWSRIDY
       ***: ..:. :***  **  ..   *:* :.*:**  .* .::**:* *. .:: ::**::******* **: *:* :*:::: :
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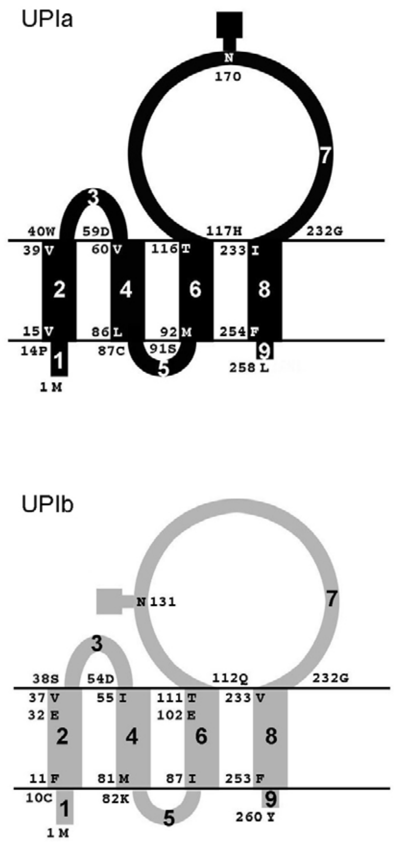
B



C

Chimera	Sequence
Ia/b1	Ib(1M-10C)+Ia(15V-258L)
Ia/b2	Ia(1M-14P)+Ib(11F-37V)+Ia(40W-258L)
Ia/b3	Ia(1M-39V)+Ib(38S-54D)+Ia(60V-258L)
Ia/b4	Ia(1M-59D)+Ib(55I-81M)+Ia(87C-258L)
Ia/b5	Ia(1M-86L)+Ib(82K-86K)+Ia(92M-258L)
Ia/b6	Ia(1M-91S)+Ib(87I-111T)+Ia(117H_258L)
Ia/b7	Ia(1M-116T)+Ib(112Q-232G)+Ia(233I-258L)
Ia/b8	Ia(1M-254F)+Ib(254Y-260Y)

Chimera	Sequence
Ib/a1*	Ia(1M-14P)+Ib(11F-260Y)
Ib/a2	Ib(1M-10C)+Ia(15V-39V)+Ib(38S-260Y)
Ib/a3*	Ib(1M-37V)+Ia(40W-59D)+Ib(55I-260Y)
Ib/a4	Ib(1M-54D)+Ia(60V-86L)+Ib(82K-260Y)
Ib/a5*	Ib(1M-81M)+Ia(87C-91S)+Ib(87I-260Y)
Ib/a6*	Ib(1M-86K)+Ia(92M-116T)+Ib(112Q-260Y)
Ib/a7*	Ib(1M-111T)+Ia(117H-232G)+Ib(233V-260Y)
Ib/a8	Ib(1M-232G)+Ia(233I-254F)+Ib(254Y-260Y)
Ib/a9	Ib(1M-253F)+Ia(255Y-258L)
Ibm*	Ib(N131R)
IbCA*	Ib(C159A/C160A/C190A)

D

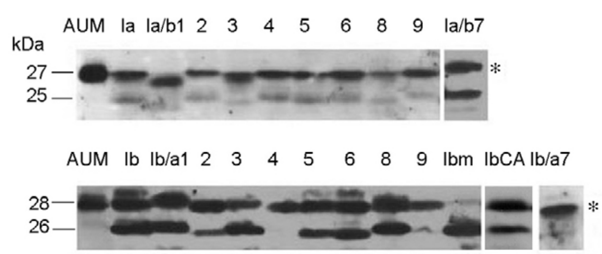


Fig. 1. Construction and expression of UPIa and UPIb chimeras. (A) Sequence alignment of bovine UPIa and UPIb using Clustal W (Thompson et al., 1994). The TM domains are indicated by solid lines. The boundaries of TM domains were defined by Yu et al. (Yu et al., 1994). The conserved Glu residues in TM1 and TM3 are indicated by shading. The amino acids Tyr-Trp-Ser in the cytoplasmic C-terminal tail of UPIb are probably part of the TM4 domain and are printed in bold typeface. (B) Schematic representations of the tetraspanins UPIa and UPIb. Protein domains are numbered 1 through 9, and the amino acids as well as the numbers characterizing their positions in the polypeptide sequence that define each domain are indicated. The tetraspanins UPIa and UPIb both have four TM domains (domains 2, 4, 6 and 8), which in the tetraspanin literature are also referred to as TM1, TM2, TM3 and TM4, respectively. They have a small cytoplasmic loop (domain 5) linking TM2 and TM3 and two exoplasmic loops (domains 3 and 7). In both UPIa and UPIb, domain 7 is much larger than domain 3, and bears an N-linked oligosaccharide, as indicated by the square connected to the loop in the diagram. N- and C-termini of both tetraspanins are exposed at the cytoplasmic side of the membrane (domains 1 and 9). (C) Tables listing the abbreviations that describe the chimeric constructs generated by swapping in a systematic fashion homologous domains between UPIa and UPIb. The following rules are followed for naming the chimeras: the abbreviation of the tetraspanin (Ia for UPIa and Ib for UPIb) from which most of the domains of the chimeric protein is derived is indicated first. Replaced domains are indicated second and abbreviated by the letter a (UPIa) or b (UPIb) followed by the number assigned to the exchanged domain as defined in B. For example Ia/b7 is constructed by substituting the large loop of UPIa (domain 7) with the corresponding one from UPIb, while leaving the other domains unchanged. The amino acid number and the one letter code abbreviation characterizing the beginning and the end of the UPIa and UPIb derived domains of each construct are also listed. Asterisks indicate the chimeric proteins that could be expressed at the cell surface. (D) 293T cells expressing the UPIa/UPIb chimeras indicated in C were analyzed by western blotting using antibodies directed against the large loop of UPIa or UPIb. All chimeras are well expressed, although the ratio of the glycosylated (*) to non-glycosylated forms varied considerably.

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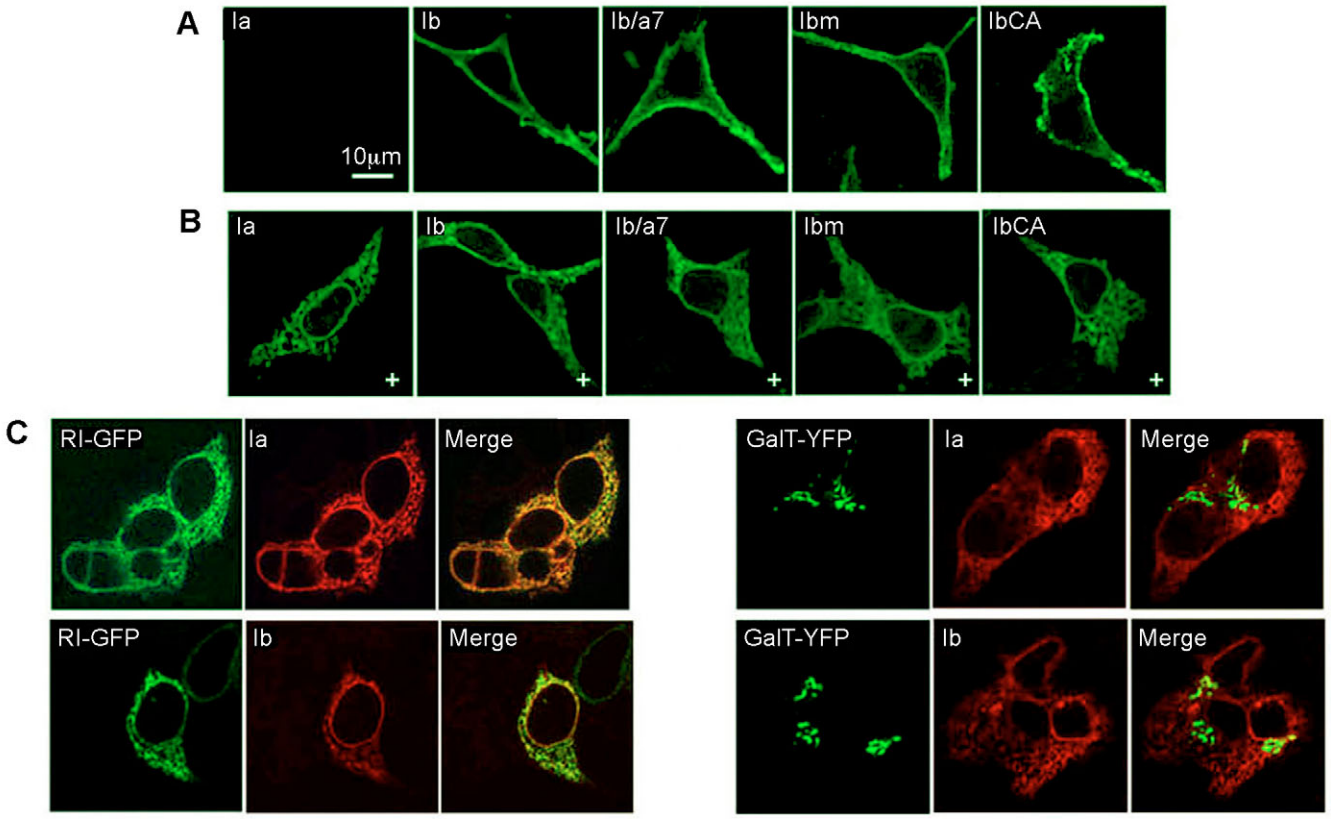

Fig. 2. Changes in the large loop of UPIb do not interfere with exit from the ER. (A) Intact 293T cells expressing UPIa, Ib, Ib/a7, Ibm and IbCA were immunostained. In contrast to UPIa, all UPIb-related proteins are expressed on the cell surface. (B) Permeabilized 293T cells expressing UPIa, Ib, Ib/a7, Ibm and IbCA showed ER staining. (C,D) To locate the intracellular UP proteins, cells were cotransfected with the ER marker Ribophorin-I-GFP (RI-GFP) or the Golgi marker galactosyl transferase tagged with YFP (GalT-YFP) and then immunostained. The secondary antibody was conjugated to Texas Red. All the intracellularly retained UP chimeric proteins were colocalized with RI-GFP. Representative images of only UPIa and UPIb are shown here.

that the hydroxyl group in Tyr is not essential for UPIb to exit from the ER. On the other hand, replacement of Tyr by Ala is not compatible with the ER exit of the UPIbA7 mutant. We also deleted stepwise the C-terminal amino acids (Fig. 3D,E), and found that the removal of the last three C-terminal amino acids did not affect the transport of the mutant proteins to the cell surface (UPIbΔ1-Δ3, Fig. 3E). However, deletion of an additional amino acid (UPIbΔ4), a highly charged Arg, which frequently defines the border of a TM domain, resulted in the complete retention of the UPIb mutant in the ER.

The C-termini of some membrane proteins containing tyrosine and phenylalanine residues have been shown to function as ER-export signals (Barlowe, 2003). To investigate whether the C-terminal portion of UPIb consisting of seven amino acids, that was originally defined as representing the cytoplasmically exposed C-terminus of UPIb (Fig. 1B) (Yu et al., 1994), had such a function, we replaced the C-terminus of the human interleukin-2 receptor alpha chain (Tac) with that of UPIa and UPIb (Tac-Ia or Tac-Ib; see Fig. 1B, Fig. 4A). Tac is a type I membrane protein that has been used extensively as a reporter to study various trafficking steps (Bonifacino et al., 1990; Letourneur and Klausner, 1992; Fu and Kreibich, 2000; Fu et al., 2000; Sevier et al., 2000). We also constructed a mutant in which the tyrosine residue in the C-terminal tail of Tac-Ib was replaced by alanine (Tac-IbA7, Fig. 4A). Immunostaining of intact cells transfected with each of the Tac-UP chimeric plasmids showed that all these three Tac-UP chimeric proteins were expressed at the cell surface (data not shown), suggesting that the cytoplasmic tail of UPIa did not function as a potent ER retention signal. To determine the rate of ER export of these Tac-UP chimeric proteins, we also expressed them in HeLa cells and performed pulse-chase experiments to assess the rate of acquisition of Endo-H resistance (Fig. 4B), which depends on the passage of the protein through the ER and Golgi compartments. While the ER exit kinetics of the chimeric proteins Tac-Ia, Tac-Ib and Tac-IbA7 were statistically indistinguishable (P>0.05) up to 120 minutes of chase, after 3 hours, the maturation rate of Tac-Ib was slightly faster than that of Tac-Ib7A and Tac-Ia (Fig. 4B,C). These results indicated that the C-terminal sequence could not explain the complete retention of the UPIb/a9 chimera in the ER (Fig. 3A,C). A more likely explanation is that Tyr254 is part of the fourth TM domain of UPIb (see below), and the cytoplasmic tail of UPIb contains only four amino acid residues (–Arg257-Ile258-Asp259-Tyr260), with Arg257 representing the halt-transfer signal that may also function in the proper positioning of TM domain 4 in the lipid bilayer.

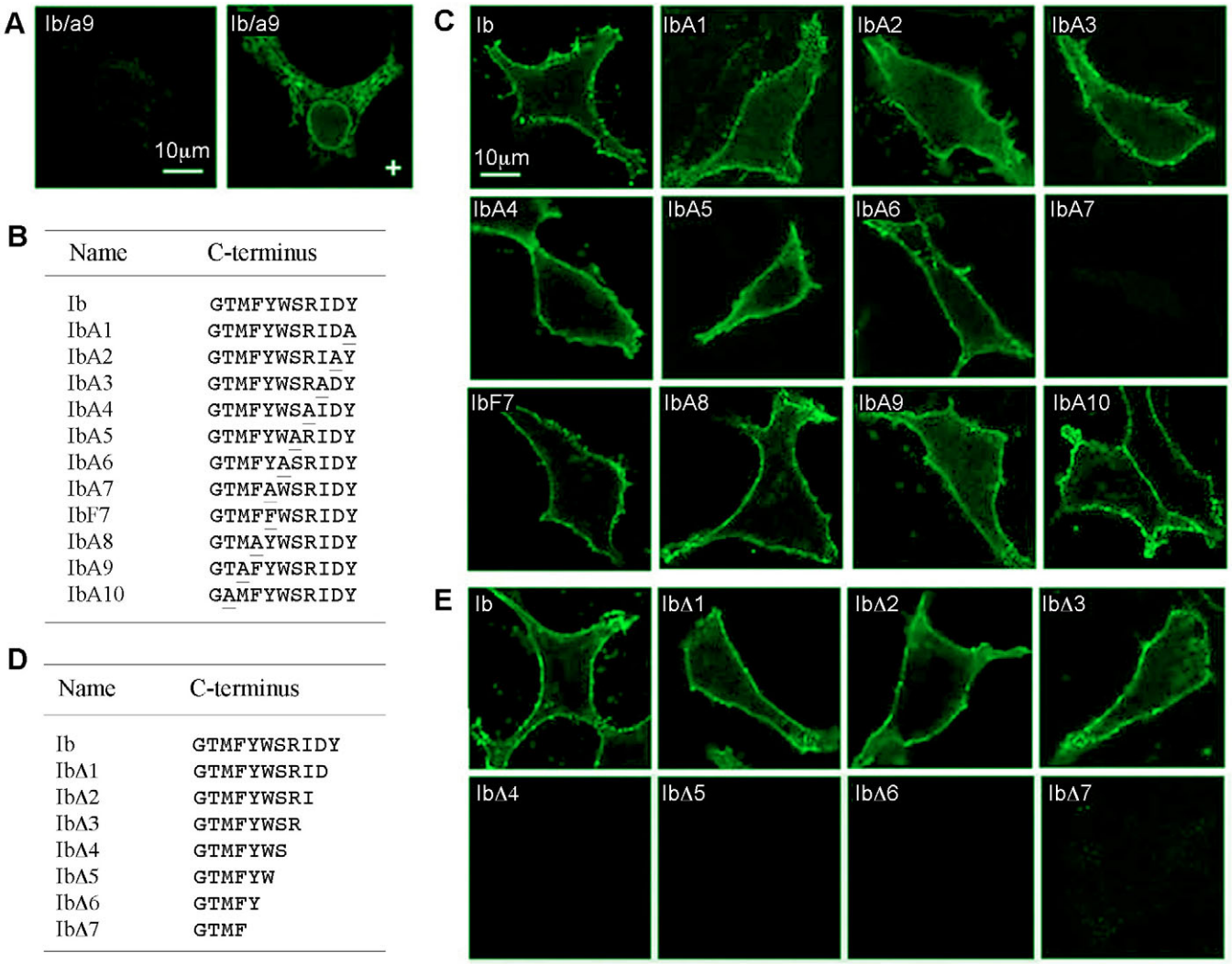

Fig. 3. Amino acids of the C-terminal domain of UPIb, including a crucial tyrosine residue, are tested for their function as an ER exit signal. (A) 293T cells expressing UPIb/a9, a chimeric protein in which the cytoplasmic C-terminal domain was replaced with that of UPIa. No immunostaining was observed in intact cells. (B) An alanine scan was performed on the C-terminal domain of UPIb, generating the Ala mutants UPIbA1-10. Similarly, Tyr253 was replaced with phenylalanine (UPIbF7). (C) Non-permeabilized 293T cells expressing the mutant proteins listed in B were immunostained. (D) Deletion mutants of UPIb were constructed by removing step-wise seven amino acids of the C-terminal domain 9. (E) 293T cells were transfected with UPIb or the deletion mutants indicated in D, followed by immunostaining of the non-permeabilized cells with a polyclonal anti-UPIb antibody. Only the deletion mutants that retain at least four amino acids of the C-terminal domain exit from the ER and are expressed at the cell surface. Deletion mutants IbΔ4 to IbΔ7 were retained in the ER as could be seen by immunostaining of permeabilized cells (data not shown).

Specific-sequence information that allows UPIb to exit from the ER resides in the transmembrane domains

Using immunofluorescence microscopy to stain the uroplakins on non-permeabilized cells, we analyzed the ability of the entire set of UPIb/UPIa chimeras (Fig. 1C) to be expressed at the cell surface. Transient expression in 293T cells showed that chimeras Ib/a2, Ib/a4 and Ib/a8, in which the TM1, TM2 and TM4 of UPIb were replaced by the corresponding ones of UPIa, failed to express at the cell surface (Fig. 5A). Western blot analysis demonstrated normal expression levels of the chimeric proteins (Fig. 1D), but instead of being targeted to the cell surface, these proteins accumulated in the ER (data not shown). This indicated that the integrity of TM1, TM2 and TM4 was crucial for UPIb to exit from the ER.

Several studies have highlighted the involvement of charged amino acids in the TMs in the process of subunit assembly and ER retention (Klausner et al., 1990; Schneider, 2004). Since both TM1 and TM3 of UPIa and UPIb contain a glutamic acid residue, which is highly conserved in tetraspanins (Stipp et al., 2003), we mutated these Glu residues of UPIb to either Ala or Gln (TM1, UPIbE32A and UPIbE32Q; TM3, UPIbE102A and UPIbE102Q; Fig. 5B). Although the Glu in TM1 appeared unimportant for ER exit because its replacement by Ala or Gln did not affect the UPIb exit from the ER, the Glu→Ala mutation in the third TM domain (IbE102A) resulted in the complete ER retention of the UPIb mutant (Fig. 5B). Interestingly, replacement of this charged Glu residue by the hydrophilic, but uncharged Gln

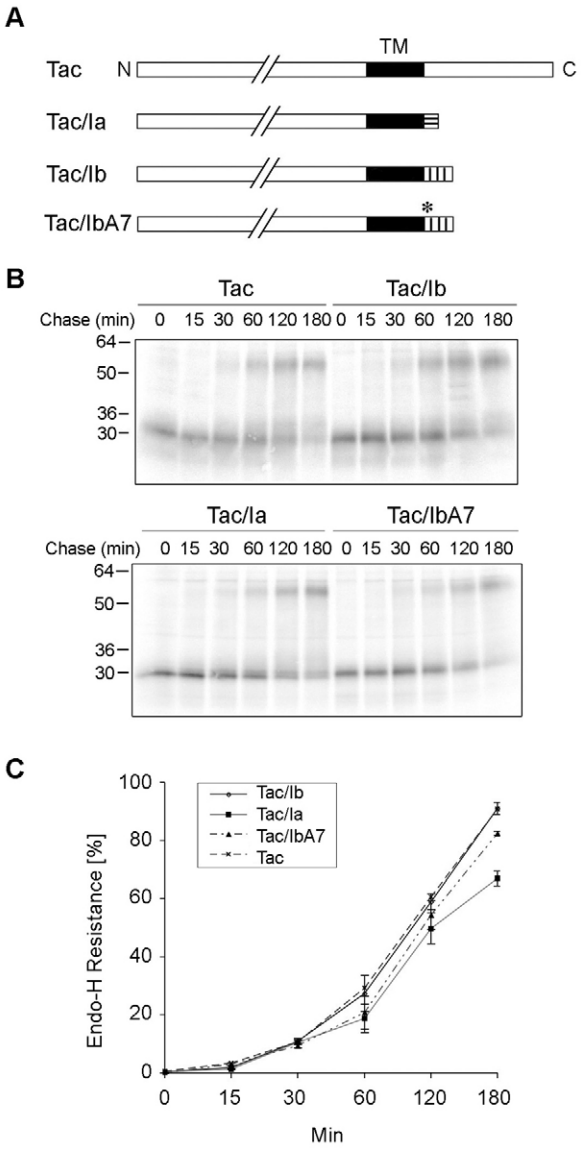

Fig. 4. Kinetics of ER exit of Tac/UP chimeric proteins. (A) Schematic drawing of the Tac antigen, a type I transmembrane protein and of Tac/UP chimeric proteins. (B) HeLa cells expressing Tac/UP chimeric proteins were pulse-labeled with [^{35}S]methionine for 15 minutes and then chased for 0 to 3 hours. The total cell lysates were harvested and subjected to immunoprecipitation using an anti-Tac antibody, followed by Endo H treatment. The Endo-H-treated samples were analyzed by SDS-PAGE and autoradiography. (C) The percentage of cells showing Endo-H resistance was calculated from densitometry values. The experiments were repeated three times to calculate s.e.m., which are indicated by the error bars. Statistical analysis was done using statistical computing software R 2.3.1.

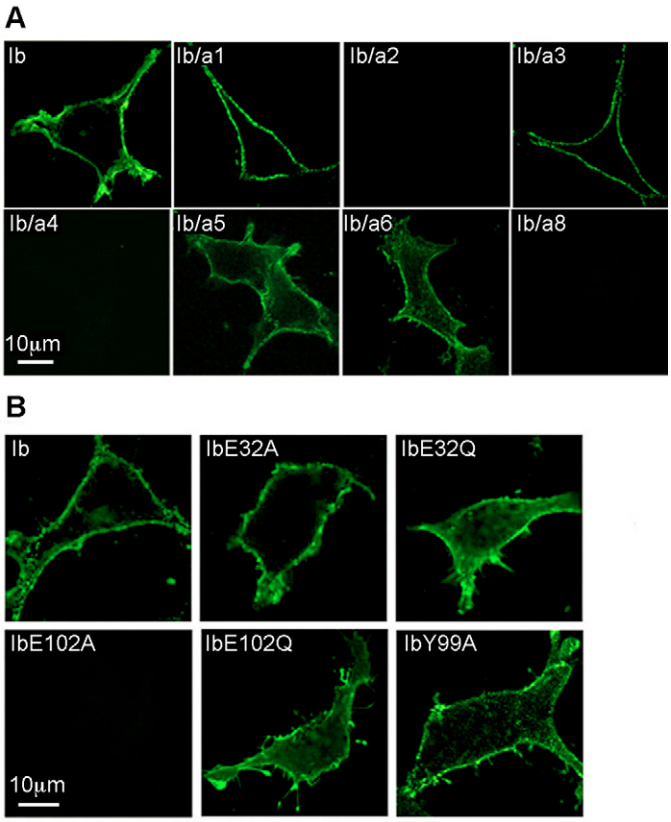

Fig. 5. Changes in any of the four TM domains prevent UPIb from exiting from the ER. (A) Non-permeabilized 293T cells expressing UPIb, UPIa or the UPIa/UPIb chimeras indicated were analyzed by immunofluorescence microscopy. The chimeras Ib/a2, Ib/a4 and Ib/a8 were not transported to the plasma membrane, but retained in the ER (not shown). (B) Point mutants of UPIb, where the Glu32 or Glu102 were replaced by Ala (IbE32A, IbE102A), or Gln (UPIbE32Q, Ib102EQ), respectively, and Tyr99 by Ala (IbY99A), were expressed in 293T cells, followed by immunostaining of non-permeabilized cells. IbE102A was not able to reach the cell surface, indicating in addition to TM1, TM2 and TM4, the glutamic acid residue in TM3 is important for the proper assembly of the TM helices of UPIb.

residue did not interfere with the surface expression of the UPIb mutant (Fig. 5B).

UPIa, as well as certain UPIb mutants form large aggregates that are unable to exit from the ER

To test the possibility that aggregation of UPIa causes its ER retention, we transfected COS-1 cells with cDNAs encoding UPIa or UPIb and fractionated the detergent extracts of the total membranes using glycerol gradient centrifugation. The distribution of UPIa (Fig. 6A) and UPIb (Fig. 6B) across the gradient was then assayed by western blotting. We found that UPIb sedimented in the middle of the gradient, with a peak in fractions 5 and 6 corresponding to ~340 kDa, which may represent the size of a functionally competent complex of UPIb that is capable of exiting from the ER. By contrast, most of the UPIa sedimented as larger-sized aggregates near the bottom of the gradient (Fig. 6A); this aggregation may account for the fact that UPIa is not able to exit from the ER. To assess the validity of this interpretation, we took advantage of our earlier observation that, when UPIa was coexpressed with UPII, the two uroplakins formed a heterodimer that is able to exit from the ER (Tu et al., 2002). We therefore tested whether this UPIa/UPII heterodimer formation prevented UPIa aggregation. Indeed we found that, when UPIa was coexpressed with UPII

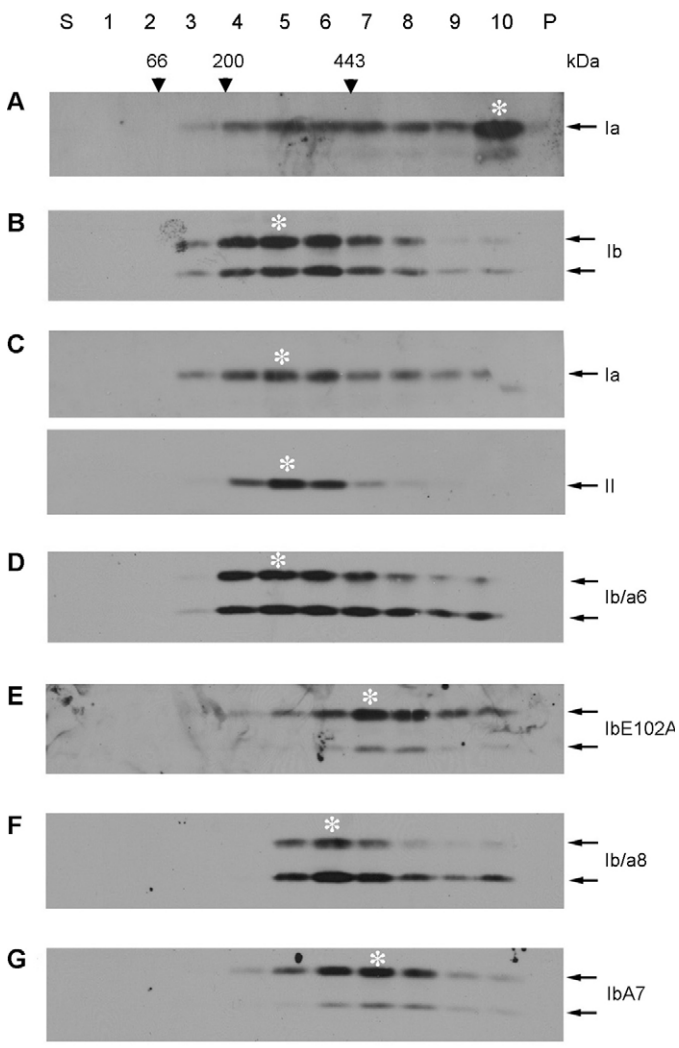

Fig. 6. In contrast to UPIb, UPIa forms large aggregates in the ER, and UPIb mutants that are unable to exit from the ER sediment faster than the wild-type UPIb. Total membrane fractions from 293T cells expressing UPIa (A), UPIb (B), UPIa and UPIb (C), UPIba6 (D), UPIbE102A (E), UPIba8 (F), or UPIbA7 (G) were extracted with digitonin (1.5%) in the presence of 0.5 M NaCl. Supernatant fractions obtained after differential centrifugation were layered onto glycerol gradients (8-30%) and after centrifugation (150,000 ***g*** for 15.5 hours), aliquots of the ten gradient fractions, the sample loading zone (S) and the pellet (P) were analyzed by western blotting. Antibodies against UPIa (A,C), UPIb (B,D-F) and UPII (C) were used to localize uroplakins in the glycerol gradient. Arrowheads in A indicate the sedimentation positions of albumin (66 kDa), amylase (200 kDa) and apoferritin (443 kDa) that were used to calibrate the gradient. Asterisks indicate the peak fractions of the sedimented UP-related proteins fractions.

(Fig. 6C), the two uroplakins co-sedimented at a position similar to that of UPIb (Fig. 6B). Interestingly, the chimeric protein UPIb/a6, which contains TM3 of UPIa and had retained its surface-expression property (Fig. 5A; Ib/a6), had a similar sedimentation profile as UPIb (Fig. 6B,D). By sharp contrast, all the UPIb mutants that were retained in the ER showed a significant shift of the peak fractions towards the bottom of the gradients (~400-480 kDa). These mutations or chimeras include IbE102A, in which the Glu102 in TM3 of UPIb was replaced by Ala (Fig. 5B, Fig. 6E), Ib/a8, with TM4 replaced by the corresponding one of UPIa (Fig. 5A, Fig. 6F), and IbA7, in which the Tyr residue close to the C-terminus of UPIb was replaced by Ala (Fig. 3C, Fig. 6G).

Discussion

The luminal and cytoplasmic domains do not play a role in determining the ER exit or retention of UPIb

Our investigation of cells expressing UPIb mutants yielded several surprising results that shed light on the assembly of tetraspanins in general, and also on the mechanisms that affect the interactions among transmembrane helices of tetraspanins. Since the deletion of the N-glycosylation site from UPIb does not result in its ER retention, N-glycosylation is not needed for the proper folding of the UPIb large loop. This finding also suggests that it is unlikely that well-known chaperones calnexin and calreticulin participate in the folding of the luminal domain of UPIb, because their chaperone function depends on their binding to the glycans of their substrate proteins (Hammond et al., 1994; Ellgaard and Helenius, 2003). In addition, elimination of disulfide bridges does not interfere with the ER exit of UPIb, indicating that they may not be required for folding of the large loop. This is consistent with studies on CD151 where alterations of the Cys motifs did not change the cellular distribution of the protein (Berditchevski et al., 2002). A possible function of the disulfide bridges in UPIa and UPIb may be to prevent unfolding when the proteins are exposed to the harsh environment of the urine. Taken together, these results demonstrate that the small and large loops of UPIb can tolerate significant modifications, to the extent that even the replacement of the entire extracellular loops by their equivalents in UPIa had no effects on UPIb exit from the ER.

An alanine scan and deletion experiments affecting the cytoplasmically exposed C-terminus (TMF**Y**WSRIDY$_{260}$) raised the interesting possibility whether Tyr254 is a part of an ER-export signal (Barlowe, 2003), since short hydrophobic motifs containing amino acids with aromatic side chains have been shown to serve as ER-export signals (Dominguez et al., 1998; Sevier et al., 2000; Otte and Barlowe, 2002). Our pulse-chase experiments on Tac-UPIb chimeras revealed, however, that this domain represented at best a weak ER-export signal (Fig. 4). Considering the rather hydrophobic character of Tyr or Trp residues (White, 2003; Hessa et al., 2005), a more plausible interpretation of these results is that Tyr254 and Trp255 actually form part of the fourth TM domain of UPIb, and that the mutation of the Tyr located close to the cytoplasmic edge of the lipid bilayer could conceivably affect the positioning of TM4 in the membrane. Therefore, all the chimeras and point mutations that we have found to affect the ER exit of UPIb actually represent modifications of the TM domains. The finding that sequence information contained in all four TM domains of UPIb can affect UPIb ER exit is most compatible with the idea that the TM domains interact with one another in a very specific fashion by forming tightly packed helix bundles (Kovalenko et al., 2005; Min et al., 2006; Seigneuret, 2006). The importance of the TM domains is supported by the fact that the amino acid sequences of the TM domains of UPIb from different species are more conserved than the loop domains (Garcia-Espana et al., 2006).

Furthermore, the TM domains of tetraspanin orthologs in different species, as well as different tetraspanins expressed in the same species, exhibit also the highest level of homology in their TM domains (Bowie, 1999; Hemler, 2001; Stipp et al., 2003). Properly bundled TM domains may allow the extracellular loops to assume a proper conformation that is monitored by the quality control system of the ER lumen.

The TM domains of UPs are tightly packed

Within each uroplakin pair (UPIa/UPII and UPIb/IIIa), there are a total of five TM domains. Our cryo-EM studies of the 16-nm uroplakin particles yielded a 3D map at 6 Å resolution, enabling us to determine the positions of the α-helices corresponding to these five TM domains (Min et al., 2006). It consists of six inner- and six outer-domains that are formed by the heterodimer pairs UPIa/UPII and UPIb/UPIIIa, respectively (Min et al., 2003). The five TM helices of each heterodimer are tightly packed. In contrast to the CIC chloride channel, whose α-helix-forming TM domains associate loosely with one another and traverse the lipid bilayer at various angles (Dutzler et al., 2002), the TM domains of uroplakins appear to traverse the membrane almost vertically. The largely parallel arrangement of the α-helices facilitates the close interactions along the entire lengths of the α-helices of the several TM domains, and may involve 'knobs-into-holes' packing (Langosch and Heringa, 1998; Zhou et al., 2000; Gratkowski et al., 2001). This may explain why the exchange of Tyr254 for Phe, another aromatic amino acid with a similar side chain volume, does not affect the ER exit of UPIb, presumably by preserving proper helix packing. By contrast, replacement of this tyrosine by alanine, which has a much smaller, aliphatic side chain, may perturb the interactions among the TM domains, resulting in the ER retention of the tetraspanin.

A highly conserved feature of tetraspanins is the presence of several potentially charged amino acid residues, including Glu in TM1 and TM3. Although charged amino acids in TM domains are energetically unfavorable for the TM helices to insert into the lipid bilayer, inter-helical hydrogen bonding has been shown to stabilize α-helical bundles of membrane proteins (Zhou et al., 2000; Gratkowski et al., 2001). For UPIb, we have demonstrated that a single mutation of Glu102 to Ala prevented UPIb from exiting the ER, whereas a change to Gln was tolerated. A Glu or Gln residue in TM3 may, therefore, be involved in forming such inter-helical hydrogen bonds, although the proximity of these residues to the interface of the lipid bilayer could also allow hydrogen bonding with head groups of lipid molecules. In fact, a Glu or Gln at this position is highly conserved throughout the tetraspanin family, supporting a crucial sequence-specific role of this residue in maintaining the helix bundle of all tetraspanins. A sequence comparison of the TM domains of UPIa and UPIb shows that the third TM domain has the highest level of identity (64%). This may explain why, of all the TM domains, only the replacement of TM3 in UPIb with the corresponding one from UPIa resulted in a chimera (UPIb/a6) that could still exit from the ER (Fig. 5B). However, replacement of Glu by Ala (UPIbE102A) interfered with ER exit, supporting the stringent structural requirements of this TM domain for the proper assembly of TM domain bundles. Tyr99, located three residues upstream could in principle form a hydrogen bond with Glu102. Expression of UPIbY99A at the cell surface demonstrated, however, that the formation of an intra-helix hydrogen bond between Glu102 and Tyr99 plays no role in allowing UPIb to exit from the ER (Fig. 5B).

Possible mechanisms for ER retention of UPIb carrying mutations in the TM domains

Recent studies have addressed the problem of ER quality control mechanisms that monitor the assembly of the TM domains buried in the hydrophobic environment of ER membranes (Ellgaard and Helenius, 2003; Krebs et al., 2004). The fact that modifications of TM domains, but not of the extracellular loops of UPIb, resulted in the ER retention of UPIb mutants raises the question of the molecular mechanisms that can recognize misalignments of the TM helices. Recent studies on the tetraspanin CD82 (Cannon and Cresswell, 2001) and the non-glycosylated membrane protein, proteolipid protein (PLP) (Swanton et al., 2003) suggest that calnexin is a part of the quality control system that detects improper assembly of their TM domains. We have performed co-immunoprecipitation experiments, similar to those described for PLP (Swanton et al., 2003), to see whether calnexin would also recognize a mutation in TM4 of UPIb, in which Tyr254 was replaced by an Ala. Although we found no selective binding of this membrane-anchored chaperone to the mutant form of UPIb (Tu et al., 2002) (data not shown), we could not rule out the possibilities that the UPIb mutant interacts with other chaperone-like proteins that recognize improperly assembled TM domains (Schamel et al., 2003; Kota and Ljungdahl, 2005).

In a recent report the behavior of mutated forms of the secretory protein transthyretin (TTR) was investigated with regard to their stability, ER export competence and formation of aggregates using mainly kinetic and thermodynamic parameters (Sekijima et al., 2005). Similar techniques to determine the secretory aptitude of TTR mutants should be ultimately applied to the study of the ER-exit behavior of the UPIb mutants. At this point the experimental approaches cannot be applied to membrane proteins. However, we were able to establish a correlation between aggregate formation of several UPIb mutants and their abilities to exit from the ER.

Our finding, that UPIa aggregate formation can be suppressed by coexpressing UPII, suggests that aggregate formation is indeed a likely cause for the ER retention of UPIa. This interpretation is supported by our observation that all our UPIb mutants tested that carry modifications in the TM domains causing ER retention sedimented faster than the native UPIb (Fig. 6). Although the formation of large aggregates may account for the ER retention of UPIa, it is also possible that the improperly folded UPIb mutants bind to as yet unidentified chaperone-related proteins leading to their ER retention. We have shown that both UPIb and the UPIa/UPII heterodimeric complex presumably form functional complexes of about 340 kDa, although the actual size could be somewhat affected by their molecular shape. Since we were unable to identify proteins that could be co-immunoprecipitated with UPIb (Tu et al., 2002), it is possible that UPIb forms a homo-oligomer. With a molecular weight of 28 kDa, about 12 molecules of UPIb could conceivably form such a ~340-kDa UPIb complex. Similarly, the UPIa/UPII heterodimer formation in the ER (Tu et al., 2002) may in fact represent the assembly of a higher order oligomer. It will be important to determine whether these

complexes of ~340 kDa are in fact assembled in the ER or whether their formation is a post-ER event. Ultimately these results have to be interpreted in the context of how uroplakin crystals containing all four major uroplakin subunits are formed, and how they relate to our finding that in the case of UPIb all four TM domains contain stringent sequence information required for the formation of a highly specific helix-bundle structure that is necessary and sufficient for UPIb to exit from the ER. It remains to be seen whether UPIa and UPIb represent prototypes of two classes of tetraspanins: one class that needs to form a heterodimer with a single-spanning membrane protein partner in order to exit from the ER and another class that does not.

Materials and Methods

cDNA constructs

The two plasmids containing the full-length cDNA encoding bovine UPIa (UPIa-pcDNA3.0) or UPIb (UPIb-pcDNA3.0) described previously (Tu et al., 2002), were used as templates to generate chimeric constructs, as well as the deletion and point mutations using classical molecular cloning technique. The boundaries of the four TM domains were determined according to the hydrophobicity plot as described (Yu et al., 1994). Alignment of the amino acid sequences of the TM domains of UPIa and UPIb showed some discrepancies. We, therefore, made adjustment of the TM boundaries at the C-terminal of TM3, where the original alignment had a four-amino-acid overhang for UPIb. Tac is human interleukin-2 receptor alpha chain. The plasmids Tac/Ia-pcDNA3.0, Tac/Ib-pcDNA3.0 and Tac/IbA7-pcDNA3.0 were constructed using a template previously described (Fu and Kreibich, 2000). The plasmid containing the HA-tagged bovine UPII cDNA was also previously described (Tu et al., 2002). A construct containing a cDNA encoding galactosyltransferase fused to yellow fluorescent protein (GalT-YFP) was a gift from Jennifer Lippincott-Schwartz (Ward et al., 2001). RI-GFP, a construct with ribophorin I cDNA inserted into pEGFPN3, was a gift from Anderi Nikonov from our laboratory.

Antibodies

Antibodies against UPIa and UPIb were raised using the fusion proteins containing the large loops of UPIa and UPIb as antigens (Tu et al., 2002). 7G7B6, a monoclonal antibody against the human Tac antigen was purchased from Ancell Corporation. The anti-HA antibody (16B12) was purchased from Berkeley Antibody Company (Richmond, CA).

Transient transfections

293T cells, HeLa cells or COS-1 cells were seeded 18 hours before transfection in six-well plates. Cells were transfected using the Fugene 6 reagent (Roche). A mixture of DNA and FuGENE6 (Roche; 1:3, w/v) in serum-free DMEM was added to cells after incubation at room temperature for 30 minutes. Cells were analyzed 24 hours post-transfection.

SDS-PAGE and western blotting

Transfected cells were rinsed with ice-cold PBS and lysed on ice in RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% sodium deoxylcholate, 1% NP-40, 0.1% SDS, 10 μg/ml aprotinin, 10 μg/ml leupeptin and 1 mM PMSF). Cell lysates were centrifuged (13,000 ***g*** for 10 minutes at 4°C) and the supernatants were stored at –20°C or used for experiments directly. Protein samples were resolved by SDS-PAGE, followed by western blot analysis (Tu et al., 2002).

Immunostaining

Cells were plated on coverslips 18 hours before transfection. 24 hours post-transfection they were fixed with 3% paraformaldehyde and blocked with 3% non-fat milk in PBS (pH 7.5). Some samples were permeabilized by adding 0.05% saponin to the blocking solution. Fixed cells were incubated with a first antibody at 37°C for 60 minutes and then with a secondary antibody (FITC-conjugated donkey anti-rabbit IgG, Jackson ImmunoResearch Laboratories). Immuno-stained cells were examined using Carl Zeiss Laser Scanning Microscope LSM510 (version 3.2) under an objective of 100×/1.4 oil DIC.

Metabolic labeling of cells

24 hours post transfection, cells were first incubated with methionine-free DMEM (Mediatech) containing 5% dialyzed fetal bovine serum for 30 minutes and then pulse-labeled with 150 μCi [^{35}S]methionine (MP Biomedicals) for 10 minutes at 37°C. Then cells were washed with serum-free medium and chased for the indicated time with complete DMEM containing 5 mM methionine.

Immunoprecipitation and endoglycosidase treatment

Following metabolic labeling, cells (2×10^6) transfected with cDNAs encoding Tac-related proteins were lysed in 0.5 ml of TBS containing 1% NP-40, 0.5% DOC. The total cell lysates were centrifuged (13,000 ***g*** for 10 minutes at 4°C) and the supernatants were incubated overnight with the first antibody (1:200) at 4°C. The complexes were precipitated by adding 25 μl of protein G-agarose beads (Roche) to the lysates and incubated for 60 minutes at 4°C. The beads were sedimented (5000 ***g*** for 5 minutes at 4°C) and washed three times with ice-cold lysis buffer. The samples were resuspended in SDS loading buffer (2% β-mercaptoethanol) and denatured by boiling for 5 minutes. The immunoprecipitates were treated with Endo-H (Roche) in 50 mM sodium citrate, pH 5.5, 1% octylglucoside, 0.05% NaN_3, 10 mM EDTA, 1 mM PMSF at 37°C for 16 hours. All samples were then subjected to SDS-PAGE and autoradiography. Quantification was done in PhosphorImager 425 (Molecular Dynamics). Statistical analysis was done using statistical computing software R 2.3.1.

Sedimentation analysis of uroplakins from digitonin-solubilized cell extracts

Although 293T cells showed the same results, we chose COS-1 cells for sedimentation analysis because they showed better adherence to the culture dishes. The sedimentation behavior of UPIa, UPIb or mutated forms of UPIb expressed in COS-1 cells was analyzed on glycerol gradients as previously described (Kelleher et al., 1992; Nikonov et al., 2002). Cells grown on 150-mm cell culture dishes were washed three times with ice-cold washing buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM $MgCl_2$, protein inhibitor cocktail (PIC) containing 0.1 μg/ml of each pepstatin A, chymostatin, leupeptin, antipain and 1 μg/ml of aprotinin). Cells were scraped into 1.1 ml homogenization buffer using a rubber policeman (20 mM Tris-HCl, pH 7.4, 500 mM NaCl, 1.5% digitonin, 1 mM $MnCl_2$, 1 mM $MgCl_2$, 1 mM DTT, PIC and 1 mM PMSF) and passed 15 times through a ballbearing homogenizer (clearance 8 μm). After incubation on ice for 30 minutes, the homogenates were centrifuged for 15 minutes at 160,000 ***g***, 4°C, using a 75Ti rotor (Beckman). 1.05 ml of the supernatant fractions were layered onto continuous glycerol gradients (8-30%) containing 20 mM Tris-HCl, pH 7.4, 50 mM NaCl, 0.125% digitonin, 25 μg/ml egg yolk L-lecithin, 1 mM $MnCl_2$, 1 mM $MgCl_2$, 1 mM DTT, PIC and 1 mM PMSF. Stock solutions of digitonin (Calbiochem) and egg yolk L-lecithin (Calbiochem) were prepared exactly as previously described (Kelleher et al., 1992). The gradients were centrifuged for 15.5 hours at 150,000 ***g***, 4°C, using a SW41 rotor (Beckman). After collecting the first 1.05 ml of the gradient (S, the loading zone), the rest of the gradient was fractionated into ten 1.05-ml fractions. Possible pellets (P) formed during centrifugation were resuspended in 1.05 ml washing buffer. 50 μl S fraction and each glycerol gradient fraction, as well as 5 μl P fraction were analyzed by SDS-PAGE, followed by western blot analysis. The sedimentation behavior of the marker proteins bovine serum albumin (66 kDa; Sigma, A8531), amylase (200 kDa; Sigma, A8781) and apoferritin (443 kDa; Sigma, A3660) was established under identical conditions, and 20 subfractions were analyzed by SDS-PAGE. The position of these proteins in the gradient was used to estimate the sizes of the UP-related complexes.

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