Uroplakin IIIb, a urothelial differentiation marker, dimerizes with uroplakin Ib as an early step of urothelial plaque assembly

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Urothelial plaques consist of four major uroplakins (Ia, Ib, II, and III) that form two-dimensional crystals covering the apical surface of urothelium, and provide unique opportunities for studying membrane protein assembly. Here, we describe a novel 35-kD urothelial plaque-associated glycoprotein that is closely related to uroplakin III: they have a similar overall type 1 transmembrane topology; their amino acid sequences are 34% identical; they share an extracellular juxtamembrane stretch of 19 amino acids; their exit from the ER requires their forming a heterodimer with uroplakin Ib, but not with any other uroplakins; and UPIII-knockout leads to p35 up-regulation, possibly as a compensatory mechanism. Interestingly, p35 contains a stretch of 80 amino acid residues homologous to a hypothetical human DNA mismatch repair enzyme-related protein. Human p35 gene is mapped to chromosome 7q11.23 near the telomeric duplicated region of Williams-Beuren syndrome, a developmental disorder affecting multiple organs including the urinary tract. These results indicate that p35 (uroplakin IIIb) is a urothelial differentiation product structurally and functionally related to uroplakin III, and that p35–UPIb interaction in the ER is an important early step in urothelial plaque assembly.

Introduction

The cell surface plays a key role in mediating the interactions between a cell and its environment. Thus, membrane proteins serve as receptors for many extracellular signals provided by growth factors, hormones, ECM, and neighboring cells. Cell surface proteins also perform specialized cellular functions including absorption, secretion, and the formation of a permeability barrier. Many of these cell surface molecules are complexes of several subunits that must be assembled, processed, and targeted to the proper domains of the cell surface in order to function. Defects in delivering functional membrane proteins to the cell surface can have major biological consequences, including compromised cellular functions or even uncontrolled cell growth leading to neoplasm. Therefore, it is important to better understand membrane protein assembly and targeting.

Urothelial plaques (also known as asymmetric unit membranes [AUM]) are structurally unique in that they consist of 16-nm protein particles arranged hexagonally, forming two-dimensional crystals (Hicks and Ketterer, 1969; Vergara et al., 1969; Brisson and Wade, 1983; Walz et al., 1995; Kachar et al., 1999). These plaques represent the major differentiation products of mammalian urothelia; thus, they can be readily isolated with an extraordinary yield of 10–20 mg of highly purified two-dimensional crystals from ~100 bovine bladders within 2 d (Wu et al., 1990). Consistent with their crystalline structure, urothelial plaques have a relatively simple protein composition consisting of four known uroplakin (UP)* subunits, i.e., UPIa (27 kD), UPIb (28 kD), UPII (15 kD), and UPIII (47 kD); Wu and Sun, 1993; Lin et al., 1994; Yu

*Abbreviations used in this paper: CD, conserved domain; MMR, mismatch repair enzyme; UP, uroplakin; WBS, Williams-Beuren syndrome.
et al., 1994; Sun et al., 1999). Cultured urothelial cells form stratified cell layers and continue to synthesize uroplakins, which, however, fail to assemble into two-dimensional crystals, thus providing unique opportunities to study the regulation of membrane assembly (Surya et al., 1990). Uroplakins Ia and Ib have four transmembrane domains, are ~40% identical in their amino acid sequences, and belong to the “tetraspanin” gene family that contains many leukocyte- and cancer-associated cell surface proteins including CD9, CD37, CD53, and CD63 (Yu et al., 1994; Maelcker et al., 1997; Hemler, 2001). On the other hand, uroplakins II and III have only a single transmembrane domain. UPII is synthesized as a precursor containing a signal peptide and a pro-sequence; the mature protein anchors into the membrane via its COOH-terminal transmembrane domain (Lin et al., 1994). Uroplakin III also has a signal peptide and a single transmembrane domain that separates a long (~200 amino acids) extracellular from a short (~50 amino acids) intracellular domain (Wu and Sun, 1993). Interestingly, the two tetraspanin-type uroplakins (UPla and UPlb) are cross-linked selectively to UPII and UPIII, respectively, suggesting the existence of two separate uroplakin pairs (Wu et al., 1995). Together, these results indicate that urothelial plaques possess an excellent model for studying membrane protein assembly (Sun et al., 1999).

To understand the assembly of urothelial plaques, it is crucial to fully characterize all the protein subunits of the plaques, including some minor components that can potentially play regulatory functions. In this regard, it should be noted that highly purified bovine urothelial plaques contain, in addition to the four known uroplakins that account for >90% of the total protein mass, another protein of 35 kD (p35) that remained uncharacterized (Liang et al., 1999). In this paper, we show that p35 represents a novel urothelial differentiation marker that is homologous to uroplakin III in sequence and transmembrane topography. Interestingly, p35 also shares a stretch of 80 amino acid residues (78–157) that is ~90% identical to a portion of a human DNA mismatch repair (MMR) enzyme-related protein (the “PMSR6” gene) that was located in a region that is frequently deleted in patients with the Williams-Beuren syndrome (WBS; Nicolaides et al., 1995). The human p35 gene is mapped to chromosome 7q11.23 near a region that is frequently deleted in patients with the Williams-Beuren syndrome (WBS; Ono et al., 1995). The human p35 gene is mapped to chromosome 7q11.23 near a region that is frequently deleted in patients with the Williams-Beuren syndrome (WBS; Ono et al., 1995). The human p35 gene is mapped to chromosome 7q11.23 near a region that is frequently deleted in patients with the Williams-Beuren syndrome (WBS; Ono et al., 1995). The human p35 gene is mapped to chromosome 7q11.23 near a region that is frequently deleted in patients with the Williams-Beuren syndrome (WBS; Ono et al., 1995). The human p35 gene is mapped to chromosome 7q11.23 near a region that is frequently deleted in patients with the Williams-Beuren syndrome (WBS; Ono et al., 1995).

**Results**

**P35 is highly conserved during mammalian evolution**

As mentioned earlier, highly purified bovine urothelial plaques contained a well-resolved p35 band (Fig. 1 a, lane 1) that we cloned by partial sequencing. RT-PCR (Fig. 1 b, lane 1), and cDNA library screening. The cDNA-deduced amino acid sequence of bovine p35 (Fig. 1 c) was found to be >90% identical to those of human and mouse p35, which we also cloned, indicating a high degree of evolutionary conservation (Figs. 1 c and 2 a). Interestingly, the amino acid sequence of bovine p35 shared several features with uroplakin III. The core proteins of p35 and UPIII were both ~30 kD in size and were 34% identical (Fig. 2 b), and they adopted a similar transmembrane topography. Both p35 and UPIII were type 1 transmembrane proteins with a signal peptide and a single transmembrane domain (Fig. 2, a, b, and d). Both had a long NH2-terminal extracellular domain (~200 amino acid residues) harboring several N-glycosylation sites (one for p35 and 4 for UPIII), and a short COOH-terminal cytoplasmic domain (~50 amino acids; Fig. 2, b and d). In addition, both possessed a domain of 19 amino acid residues (190-198) encoding 279-amino acids that can account for all five partial sequences from mass spectrometry, and 1,343 bp of 3'-untranslated region. The 5' upstream sequence contained multiple stop codons, and the translational initiation site conformed to the consensus sequences (Kozak, 1984, 2000), whereas the 3'-untranslated region contained a polyadenylation signal with a poly(A) tail (not depicted). Arrowhead marks the boundary of a possible signal peptide. Dotted underline denotes a stretch of 80 amino acid residues that are homologous to the NH2-terminal region of a hypothetical human DNA MMR enzyme-related protein hPMSR6. Box denotes an amino acid sequence highly conserved in p35 and uroplakin III. Thick bar marks the hypothetical transmembrane domain.

![Figure 1. Isolation and characterization of the bovine p35 cDNA.](image)

**P35 expression is urothelial-specific and differentiation-dependent**

Northern and PCR analyses showed that p35 expression was urothelial-specific in both bovine (Fig. 3, compare a with b...
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and c) and mouse (Fig. 3, compare d with e and f). To localize the p35 protein, we generated rabbit antisera against three synthetic peptides of p35. All these antisera recognized the p35 protein. Figure 2. The amino acid sequence of p35 is conserved and is highly homologous to uroplakin III. (a) cDNA-deduced amino acid sequences of p35 from human (H), mouse (M), and bovine (B). Arrowhead marks the end of signal peptide. HPM6,6 and CD denote the HPM6,6 homologous domain, and the conserved domain shared by p35 and uroplakin III (and uroplakin II), respectively. Thick bar denotes the transmembrane domain. (b) Alignment of bovine p35 and uroplakin III. Dots were introduced to maximize the alignment. Asterisks indicate identity. Note the significant sequence homology between the two proteins, particularly the CD. (c) A part of the CD is also shared by uroplakin II (underlined). (d) Overall structure of bovine p35 and uroplakin III. Note the similarities in their size, and the location of the transmembrane and CDs. SP denotes signal peptide.

Figure 3. Urothelial specificity of p35 in bovine and mouse. For bovine tissues (a–c), 20 μg total RNA from 11 tissues was separated on a formaldehyde gel, transferred to a nylon membrane, and probed with (a) p35 cDNA, (b) β actin cDNA, (c) an ethidium bromide (EB)-stained gel. For mouse tissues, cDNAs from various tissues were used to amplify (d) p35, (e) uroplakin III, or (f) glyceraldehyde phosphate dehydrogenase as a loading control. Tissues analyzed include bladder (B), brain (Br), forebrain (FB) and rear brain (RB), colon (C), esophagus (E), heart (H), intestine (I), kidney (K), liver (L), lung (Lu), skeletal muscle (M), spleen (S), and stomach (St).

Figure 4. The urothelial differentiation-dependent expression of p35. (a) A rabbit antiserum was generated to a synthetic peptide (positions 268–279 in the cDNA-deduced protein sequence of p35) and used to immunoblot total bovine urothelial proteins (lane 2), total membrane proteins (lane 3), purified bovine urothelial plaque proteins (lane 4), and purified mouse urothelial plaque proteins (lane 5). Lanes M and 1 denote molecular mass markers (M; from top to bottom: 66, 45, 36, 29, 24, 20, and 15 kD) and total bovine urothelial plaque proteins (1). Note the strong reaction of this antibody to p35 of purified urothelial plaques, and the mono-specificity of the antiserum in total urothelial extracts. (b and c) Deglycosylation of p35 (b) and uroplakin III (c). Total bovine urothelial plaque proteins (1); same proteins after overnight incubation in the deglycosylation buffer (lane 2); same proteins treated with Endo H (lane 3) or Endo F (lane 4). The proteins were electrophoretically separated, transferred to nylon membranes, and blotted with antibodies to p35 (b) or uroplakin III (c). Note the shift of both p35 and uroplakin III to ∼30 kD, indicating similar core protein size and suggesting the presence of a complex N-linked oligosaccharide. The high molecular weight band represents dimer formation. (d–g) Immunolocalization of p35 (d and f) and uroplakin III (e and g) in bovine (d and e) and mouse (f and g) urothelium. Note the association with p35 and UPIII with superficial umbrella cells in bovine urothelium, and with suprabasal cells in mouse urothelium. (h) Differentiation-dependent expression of p35 and UPIII in cultured bovine urothelial cells. (lane 1) mouse 3T3 cells, (lane 2) bovine bladder fibroblasts, cultured bovine bladder epithelial cells at (lane 3) 50% confluence, (lane 4) 100% confluence, (lane 5) 3-d post confluence (pc) (lane 6) 6-d pc, (lane 7) 9-d pc, (lane 8) in vivo bovine bladder epithelium, and (lane 9) bovine ureteral epithelium. Bottom panel shows the ethidium bromide staining of 28
and 18s ribosome RNA as a loading control. Note the differentiation-dependent expression of both p35 and UPIII.

(ii) Immunofluorescent staining of cultured bovine urothelial cells (5-d pc, permeabilized and fixed with cold methanol/acetone) using an affinity-purified p35 antibody. Note the selective staining of superficial cells (*). Bars, 50 μm.

**Figure 5. Dimerization of p35 with uroplakin Ib, a partner of uroplakin III.**

(a) Human kidney 293T cells were cotransfected with cDNAs of p35 and one of the four uroplakins. Total cell lysates were treated with an antibody against p35, and the immunoprecipitates were immunoblotted with different uroplakin antibodies (top), or with anti-p35 (bottom). Lanes 1–3 show total cellular lysates (lane 1), immunoprecipitates from preimmune sera (lane 2), or from anti-p35 (lane 3). Note the selective coprecipitation of p35 and UPIb.

(b) 293T cells were transfected with p35 cDNA alone (lanes 1, 3, 5, and 7), or with p35 plus uroplakin Ib cDNAs (lanes 2, 4, 6, and 8). Total cellular proteins were isolated 24 h later, and immunoblotted using antibodies to p35 (top) or uroplakin Ib (bottom). The samples were then analyzed directly (lanes 1 and 4), after a 24-h incubation in a buffer (lanes 2 and 5), or in a buffer containing endoglycosidase H (lanes 3 and 6). Note that p35, when transfected alone, was Endo H sensitive, but acquired Endo H resistance when cotransfected with uroplakin Ib.

(c) 293T cells were transfected with p35 cDNA alone (lane 1), UPIb cDNA alone (lanes 2 and 3), or with both p35 and uroplakin Ib cDNA (lanes 4–13). Cells were pulse-labeled with [35S]methionine for 10 min and chased for various periods of time (marked in minutes on top). Immunoprecipitation (IP) was performed with preimmune serum (PI), anti-p35, or anti-UPIb antibodies as indicated. The precipitated products were analyzed by SDS-PAGE. The nonglycosylated (p35 or Ib) and glycosylated (p35* or Ib*) forms of the molecules are as marked. M denotes molecular mass markers (from top to bottom: 75, 50, 37, 25, and 20 kD). Note that the p35/UPIb heterodimer can be immunoprecipitated with anti-p35 immediately after pulse labeling (lane 5). (d) Immunoprecipitates prepared from cells cotransfected with p35 and UPIb cDNAs (as in panel c) were either treated with endoglycosidase H (+) or incubated
Effects of uroplakin III ablation on p35 expression

The fact that UPIb could apparently form heterodimer with both p35 and its closely related UPIII suggested that these two molecules may perform similar functions. If so, one might expect that genetic ablation of UPIII could lead to the up-regulation of p35 as a compensatory mechanism. To test this possibility, we studied the expression of p35 in UPIII-deficient mice that we generated recently (Hu et al., 2000). UPIII deficiency led to a coordinated change in the mRNA and protein levels of uroplakins within the same “pair.” Thus, within the UPIa/UPII pair, the mRNA of both uroplakins increased approximately twofold, suggesting a coordinated upregulation and/or stabilization (Fig. 7 a). However, on the protein level, these two uroplakins decreased ~20-fold in total cell lysates, suggesting lower translational efficiency and/or degradation possibly due to suboptimal subunit stabilization (Fig. 7 b). Within the putative p35/UPIIb pair, both mRNAs increased approximately sixfold (Fig. 7 a), whereas the pro-
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Proteins decreased three- to 10-fold (Fig. 7 b), indicating that p35 and UPIb were coordinately regulated. Immunohistochemical staining results showed that although much of UPIb was mis-targeted to the basal-lateral surface (Hu et al., 2000), small amounts of UPIb and p35 were associated with the apical urothelial surface (Fig. 7 c; see Discussion).

**Human p35 gene is localized on chromosome 7q11.23 near the deleted region of the WBS**

Human p35 gene was localized on chromosome 7q11.23 with six exons spanning ~5 kb (Fig. 8 a), which was 0.5 Mb centromeric to the PMSR6 gene. The sequence of p35 gene was distally homologous with uroplakin III gene (Fig. 8 a), but was highly related to PMSR6 gene that encoded a hypothetical DNA MMR enzyme-related protein (Horii et al., 1994; Nicolaides et al., 1995). The close relationship between p35 and PMSR6 gene (Fig. 8 a) was indicated by the fact that (1) a large stretch (~25 kb) of the 5' upstream sequence of p35 gene was >90% identical to the 5' half of PMSR6 gene containing its first 5 exons; (2) the p35 genomic sequence starting from the first intron to the end of third intron was >90% identical to the sixth exon of PMSR6 and its surrounding intron sequences; (3) the third exon of p35 gene, which corresponded to exon 6 of the PMSR6 gene (Fig. 8 a, arrowhead), encoded the 80 amino acid residues shared by these two proteins (Fig. 2 b); and (4) the 3' downstream sequence of p35 (>20 kb downstream from the sixth or last exon) was >90% identical to the 3' downstream sequence of PMSR6 gene (Fig. 8 b). Therefore, it seemed likely that p35 and PMSR6 genes were evolved by gene duplication (see Discussion); given the more conserved and perhaps primitive nature of the DNA MMR function, we speculate that PMSR6 gene was the precursor of p35 gene. Southern analysis of human genomic DNA using a partial human p35 cDNA probe (corresponding to the second and third exons of p35; a sequence shared by the two genes) showed two equal intensity bands in DNA digested by five different restriction enzymes, suggesting the existence of two closely related genes (p35 and PMSR6; Fig. 8 b). PCR products generated using primers based on the second and third exons of p35 gene contained the sequence of not only p35, but also that of PMSR6 (Fig. 8 c), thus confirming the close relationship between these two genes. BLAST analysis of the HTGS database showed that p35 and R6 genes are present in BAC clone RP11-340A14 and RP11-506L7, respectively. Based on Peoples et al. (2000), Valero et al. (2000), and the HTGS-derived data, these two clones are localized on chromosome 7q11.23 immediately telomeric to HIP1, a single-copy gene that maps telomeric to the frequent distal breakpoint of the WBS (OMIM 194050; see Discussion and Fig. 8 d).

**Discussion**

**An expanded uroplakin pair concept: uroplakins IIIa and IIIb (p35)**

Here, we show that p35, a novel urothelial marker, is closely related to uroplakin III in terms of the following: their core protein size (Fig. 4, b and c); their transmembrane topology (Figs. 2 and 9); their sequence homology (Fig. 2 b); their sharing of a juxtamembrane sequence of 19 amino acids (Fig. 2, b–d, and Fig. 9); their interaction with uroplakin Ib in the ER (Fig. 5); and their urothelial specificity and differentiation dependence (Figs. 3 and 4). Based on these similarities, we propose to rename UPIII and p35 as uroplakins IIIa and IIIb, respectively. As mentioned earlier, the four known uroplakins can be separated into two pairs consisting of UPIa/UPII and UPIb/UPIII according to the following data: first, chemical cross-linking using bifunctional cross-linking reagents of various length and hydrophobicity resulted in the specific cross-linking of UPIa and UPIb to UPII and UPIII, respectively (Wu et al., 1995). Second, complexes of UPIa–UPII and UPIb–UPIII can be isolated by ion exchanger chromatography from total uroplakins dissolved in octyl-glucoside (Liang et al., 2001). Third, the
knockout of mouse uroplakin III gene is accompanied by the selective mistargeting of its putative partner, UPIb (Hu et al., 2000). Our present in vitro transfection data indicate that UPIb enables both UPIIa and UPIIIb to exit from the ER and to reach cell surface (Figs. 5 and 6; Tu et al., 2002), thus broadening the pair concept to include UPIIIb which, as a structural homologue of UPIIIa, can also partner with UPIb (Fig. 9).

Dimerization of UPIIIb and Ib in ER

It is known that oligomerization of many membrane proteins takes place in the ER, and that in most instances the unassembled proteins are unable to exit from this compartment (Reddy and Corley, 1998; Ellgaard et al., 1999; Green, 1999). Our transfection experiments indicate that UPIIIb, when expressed alone, is trapped in the ER (Fig. 6 a), and that UPIb, but not any other uroplakins, can rescue UPIIIb, allowing it to exit from the ER and to reach the cell surface (Fig. 6 b). This conclusion is supported by our finding that the cotransfected UPIbs converts the N-linked oligosaccharides of UPIIIb from an Endo H–sensitive to an Endo H–resistant form (Fig. 5 b), indicating that UPIIIb enables UPIIIb to be processed by glycosylation enzymes that are known to be associated with the medial compartment of the Golgi apparatus. Immunoprecipitation studies using metabolically labeled cells showed that UPIb and UPIIIb can coperiplicate even before UPIIIb’s sugars acquire Endo H resistance, indicating that heterodimer formation must have occurred in the ER (Fig. 5, c and d). Finally, the fact that antibodies to UPIIIb selectively pulled down UPIb in metabolically labeled cells, without pulling down any other labeled proteins, suggests that UPIIIb interacts directly with UPIb without the participation of other proteins that are present in the 293T cells (Fig. 5 c). Additional studies are needed to further elucidate how the UPIIIb/UPIb and UPIIIa/UPIb heterodimers interact with the other uroplakin pair (UPII/UPIa) to form a 16-nm particle, and eventually to assembly into a two-dimensional crystal of urothelial plaque.

Possible functions of UPIIIb

Existing data suggest that UPIIIb plays a urothelial-specific function most likely involved in urothelial plaque formation. First, UPIIIb is associated with the differentiated urothelial cells (Fig. 3 and Fig. 4, d–i). Second, the amino acid sequences of human, mouse, and bovine UPIIIb are >90% identical (Fig. 2 a), suggesting that the protein plays a highly conserved biological function. Third, the fact that UPIIIb is highly enriched in purified bovine urothelial plaques suggests that it is a plaque component (Fig. 4 a). Fourth, the relatively large cytoplasmic domains of UPIIIb and UPIIIa suggest these may mediate membrane–cytoskeletal interactions serving to anchor the urothelial plaque to an underlying cytoskeletal network (Wu and Sun, 1993). The fact that UPIIa and UPIIIb exhibit a much higher degree of homology in their luminal domains (36%) than their cytoplasmic tails (18%; Fig. 2 b) suggests that their conserved luminal domains interact with a common target, i.e., UPIb, whereas their cytoplasmic tails play chain-specific functions (Liang et al., 1999). A potentially important difference between the cytoplasmic tails of UPIIIa and UPIIIb is that only the former harbors several potential phosphorylation sites (Wu and Sun, 1993). Fifth, unlike UPIIIa that is present as a major component in purified urothelial plaques, UPIIIb is present at a much lower level amounting usually to <10% of UPIIIa (Fig. 1 a and Fig. 4 a, lane 1) and this level is somewhat variable. Recently, we have shown by EM localization that UPIIIa is associated with almost all urothelial plaques in mouse and bovine urothelial umbrella cells (Liang et al., 2001), suggesting that UPIIIa is a constant component of both cytoplasmic and apical plaques. The low stoichiometry of UPIIIb suggests that this protein is associated with a subpopulation of urothelial plaques, possibly conferring special structural or functional properties to these plaques.

A puzzling phenotype of uroplakin III knockout mice is the presence of some normal-looking (albeit much smaller) urothelial plaques (0.05–μm-diam vs. 0.2–1–μm in normal urothelium; Hu et al., 2000). There are two possible explanations for this UPIIIa-independent plaque formation. One is that the ablation of UPIIIa abolished the UPIIIa/UPIb pair; but the remaining UPII/UPIa pair can still form small urothelial plaques. Another possibility is that all four uroplakins are required for plaque formation, but a small amount of UPIIIa homologue, i.e., UPIIIb, can rescue some of the other three uroplakins and is thus responsible for the formation of the observed small plaques. Our current finding that UPIIIb is up-regulated relative to other uroplakins in the UPIIIa-deficient urothelium (Fig. 7, a and b) supports the latter interpretation.

Possible significance of the CD and hPMSR6-related domains

It is interesting that UPIIa and UPIIIb share 19 amino acid residues (IDTWPGRRSGDMIIITSIL) located proximal to their single transmembrane domain (Fig. 2, b and d). The structural significance of this conserved domain (CD) is unclear, although our preliminary data indicate that mutations in this domain do not affect UPIIIb–UPIb interaction in the ER as assayed by cotransfection (unpublished data). Additional
studies are needed to determine whether the CD plays a role in subsequent steps of asymmetric unit membrane assembly.

A surprising feature of human UPIIIb protein is that it contains a domain of 80 amino acid residues that are >95% identical to a portion of hPMSR6, a hypothetical DNA MMR enzyme–related protein. That this hPMSR6-related domain is a part of the UPIIIb molecule is supported by several findings. First, the cDNA sequence of UPIIIb has been validated by the fact that it can account for all five partial protein sequences of electrophoretically purified p35 (Fig. 1 c), and that antibodies raised against several p35 peptide recognized the correct p35 protein (Fig. 4 a). Second, sequencing of a PCR product between the hPMSR6 and CDs confirmed the colinear existence of these two domains in a single p35 cDNA. Third, the segment of 80 amino acids that are shared by P35 and hPMSR6 is encoded by a single exon (exon 2 of the p35 gene and exon 6 of the hPMSR6 gene; Fig. 8 a). Fourth, both human p35 and hPMSR6 genes are localized on chromosome 7q11.23, and the genomic structures of these two genes show striking conservations, suggesting the derivation of one from another by gene duplication (Fig. 8 a).

Because little is known about the protein encoded by the hPMSR6 gene, we do not know the functional significance of the hPMSR6-related domain in UPIIIb. However, we do know that because this domain is not present in UPIIIa, the entire R6 domain is unlikely to be essential for p35 to form heterodimer with UPIIb. DNA MMR enzymes, including hPMS2, play a key role in promoting genetic stability by repairing DNA replication errors, inhibiting recombination between nonidentical DNA sequences, and participating in cellular responses to DNA damage (Modrich and Lahue, 1996; Urban et al., 1996; Baumer et al., 1998). Because p35 gene is localized near the telomeric duplicated sequence of the WBS and because UPIIIa knockout led to widespread anomalies in the lower urinary tract, including vesicoureteral reflux (Hu et al., 2000), it is important to determine whether p35 deletion plays a role in causing some of the observed urinary tract defects in WBS.

Materials and methods

Bovine urothelial cDNA libraries

A bovine bladder–specific cDNA library was generated by suppression subtractive hybridization using a PCR-Select cDNA Subtractive Kit (CLONTECH Laboratories, Inc.; Diatchenko et al., 1996; Deng et al., 2001). Poly(A)+ mRNAs were isolated from bovine, mouse, and human bladder using a PolyATtract mRNA isolation system (Promega). Bovine, mouse, and human bladder cDNA libraries were constructed using a HybniZAP 2.1 XR library construction kit (Stratagene) and were screened using a partial p35 cDNA. P35 phagemides of p35 were obtained by the in vivo excision of a phage DNA from all positive clones. DNA sequencing was performed using the dideoxynucleotide chain termination method (Sanger et al., 1977) with a DNA sequence kit (US Biochemical Corp.).

Mass spectrometry

The Coomassie blue–stained band was excised from the gel, and the protein was digested in-gel (Shevchenko et al., 1996). Digested peptides were analyzed by LC/MS and LC/MS/MS on a mass spectrometer (API III+; MDS Scies) as described previously (Rising et al., 1993). De novo sequencing was done manually, with a complete set of b and y ions observed for most of the identified sequence.

RT-PCR and Northern and Southern blotting

Total RNAs from various tissues were isolated using an RNAgent® system (Promega). For Northern blotting, 10 μg total RNA was electrophoretically transferred onto a nylon membrane and hybridized with different 32P-labeled probes. For RT-PCR, cDNA was synthesized using a SuperScript™ first-strand synthesis system (GIBCO BRL). Primers for bovine p35 were forward, 5'-GTGCTTTAGGTTGGAATG-3', and reverse, 5'-ATCCCGTGCTTCACTCCGGCG-3'. Primers for mouse p35 were forward, 5'-ACC-TGGAAGGGAGAAC-3', and reverse, 5'-AAAGTTTCCCCATGGAGGAG-3'. 10 μg of human blood genomic DNA was digested with various restriction enzymes for 16 h, size-fractionated on an 0.8% agarose gel, transferred to a nylon membrane, and probed with a 300-bp 5' end of human p35 cDNA (Lin et al., 1994).

Generation and purification of antibodies against synthetic peptides

Three peptides, (1) VLDHSSAADYVW, (2) TNSRGSPQAETRWSD, and (3) EPGLERFSLSP were synthesized (Genemed Synthesis, Inc.) based on the cDNA-deduced amino acid sequence of bovine p35. An additional cysteine residue was placed at the COOH terminus of peptide b and NHR terminus of peptides a and c. These peptides were conjugated to keyhole limpet hemocyanin. 100 μg of each conjugated peptide was used to prime each rabbit and 50 μg for booster injections at 2-wk intervals. Antibodies
were affinity-purified using a peptide-conjugated Ultragel lodoacetamide column (Pierce Chemical Co.). The eluted antibodies were concentrated using Centricon-30 (Amicon).

**Immunoblotting and enzymic deglycosylation**

Proteins were separated by SDS-PAGE on 13% polyacrylamide gels with an acrylamide/bisacrylamide ratio of 120:1, and were electrophoretically transferred onto a nitrocellulose membrane. The membrane was briefly stained with Fast Green (Sigma-Aldrich) to reveal protein bands. After blocking with 2%nonfat milk in PBS, the blots were incubated with specific primary antibodies. The primary antibodies were detected with a goat anti–rabbit or anti–mouse IgG conjugated with HRP (ICN Biomedicals). After washing with PBS, the membrane was developed with the enhanced chemiluminescence Western blotting detection system (Pierce Chemical Co.) and exposed to Fuji x-ray film at RT. For enzymic deglycosylation, bovine urothelial plasmas were solubilized in 0.5% SDS at RT for 15 min, and then incubated with endoglycosidase H or with N-glycosidase F (Roche). Glycosidase was omitted in negative control incubation. After incubation at 37°C for 16 h, the reaction mixtures were resolved by SDS-PAGE and electrophoretically transferred onto a nitrocellulose membrane. P35 was detected by immunoblotting using a peptide antibody against bovine p35.

**Urothelial cell culture**

Bovine urothelial cells were isolated from bovine bladder epithelial cells and cultured in the presence of mitomycin C–treated NIH 3T3 feeder cells in DMEM containing 15% FCS, 0.5 μg/ml hydrocortisone, 5 ng/ml cholera toxin, 5 μg/ml insulin, and 15 mM EGF as described previously (Surya et al., 1990). 3T3 feeder cells and any contaminating fibroblasts were removed by spraying the culture with 0.01% EDTA in PBS after the cell sedimented (5,000 g for 10 min at 4°C), and the supernatants were stored at −70°C for further analysis.

**Immunohistochemistry**

Paraffin sections were treated with 1% hydrogen peroxide in methanol to block the endogenous peroxidase, and incubated with normal 1:50 goat serum in 2% BSA, followed by overnight incubation at 4°C with a peptide antibody against bovine p35 and AU1, an mAb against UP3 (Liang et al., 2001; Riedel et al., 2001). Primary antibodies were detected with specific HRP-conjugated secondary antibodies (ICN). Preimmune serum and 2% BSA were used as negative controls. After counterstaining with hematoxylin, sections were mounted using glycerin gelatin and observed under a Zeiss Axiohot microscope.

**Transient transfection of 293T cells**

cDNAs of all four uroplakins and p35 were isolated by PCR using bovine urothelial cDNA as the template. Additional EcoRI and XhoI restriction sites were introduced to the forward and reverse primers, respectively. The cDNAs were cloned into the pcDNA3 vector using the EcoRI and XhoI sites (Invitrogen). An HA tag was inserted into the small extracellular loop region of the UP1b cDNA. 293T cells were maintained in DMEM supplemented with 10% FBS. The FuGENE 6 reagent (Roche) was used for transient transfection. 18 h before transfection, 293T cells were plated in 6-well plates (6 × 10⁴ cells/well). cDNA was mixed with FuGENE 6 (Roche; 1:3, wt/vol) in serum-free DMEM, incubated at RT for 30 min, and added dropwise to the 293T cells. 24 h after transfection, cells were rinsed with ice-cold PBS and lysed in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% sodium deoxycholate, 1% NP-40, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM PMSF) on ice. The total cell lysates were centrifuged at 13,000 g for 10 min at 4°C, and the supernatants were stored at −20°C or used for experiments directly.

**Metabolic labeling and immunoprecipitation**

24 h after transfection, cells were rinsed twice with PBS prewarmed to 37°C and incubated for 30 min in a methionine-free DMEM containing 0.5% dialyzed bovine serum. The cells were then pulse-labeled for 10 min with [35S]methionine (ICN Biomedicals) at 0.05 μCi/ml. Chase was done at 37°C for the indicated periods with DMEM containing an excess of unlabeled methionine (30 μg/ml). The labeled cells were washed with 4°C PBS and extracted with lysis buffer for immunoprecipitation. For immunoprecipitation, protein G–agarose beads (Roche) were preconjugated with primary antibodies or preimmune serum for 2 h at 4°C. After being precleaned with protein G–agarose beads, the supernatants of total cell lysates were incubated overnight at 4°C, with gentle agitation, with the antibodies-conjugated protein G–agarose beads. The beads were sedimented (5,000 g for 2 min) and washed four times with cold lysis buffer. The antigens were then eluted with 0.2% SDS sample buffer.

**Immunostaining**

293T cells were plated on coverslips 24 h before transfection. 24 h after transfection, the cells were fixed with 3% PFA in PBS, pH 7.5, and incubated with 5% nonfat milk in PBS. Some cells were permeabilized by adding 0.05% saponin to milk solution. The fixed cells were incubated at 37°C for 90 min with a rabbit antibody against p35 (1:100) and a mouse mAb against the HA tag of the UP1b, followed by a secondary antibody (1:200, Texas red–conjugated donkey anti–mouse IgG and FITC–conjugated donkey anti–rabbit IgG; Jackson ImmunoResearch Laboratories). Immunostained cells were scanned using a confocal microscope (model LSM510; Carl Zeiss Microimaging, Inc.).

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