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. There-fore, 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; 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; Maecker 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 (UPIa and UPIb) 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 plaque provides an excellent model for studying membrane protein assembly (Sun et al., 1999).
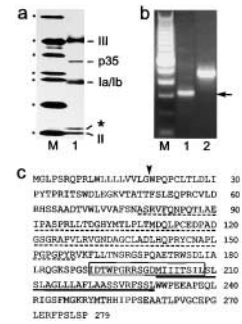
To understand the assembly of urothelial plaques, it is crucial to fully characterize all the protein subunits of the plaques, including some minor components that can potentially play regulatory functions. In this regard, it should be noted that highly purified bovine urothelial plaques contain, in 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 PMSR6 protein (Horii et al., 1994; 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; Online Mendelian Inheritance in Man [OMIM], 2002, MIM no. 194050, available at http://www.ncbi.nlm.nih.gov/omim; Francke, 1999), a developmental disorder affecting multiple systems including the urinary tract (Babbitt et al., 1979; Morris et al., 1990; Francke, 1999).

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

Figure 1. **Isolation and characterization of the bovine p35 cDNA.** (a) SDS-PAGE analysis of purified bovine urothelial plaques (lane 1) showing the presence of a 35-kD protein (p35) in addition to the four known uroplakins (Ia, Ib, II, and III). The electrophoretically purified p35 protein yielded five partial sequences by mass spectrometry: (1) (I/L)D(I/L)(I/L)PYTPR; (2) QNPQT(I/L)AE(I/L)HNTR; (3) DAGSS(I/L)AD(I/L)HQPR; (4) YMTNP(I/L)TM; and (5) SSYNAP(I/L)PGNGAVTK. M denotes molecular mass markers (from top to bottom; 66, 45, 36, 29, 24, 20, and 14 kD). Asterisk marks an uncharacterized 18-kD band. (b) Agarose gel analysis showing a 186-bp PCR fragment of p35 generated using degenerate primers designed according to two partial protein sequences of p35 (QNPQTLAE and DAGCLADLHQPR; lane 1, arrow), and a control PCR products of bovine uroplakin III (lane 2). M denotes DNA size markers. (c) The cDNA-deduced amino acid sequence of bovine p35. The 186-bp PCR product was used to screen a bovine urothelial cDNA library yielding an ∼2.4-kb full-length cDNA that contained a 198-bp 5'-untranslated sequence, a single open reading frame of 1,038-bp 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.

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-IDTW-PGRRSGDMIIITSIL-208 for p35; 197-IDTWPGRRSG-GMIVITSIL-215 for uroplakin III) that was located in a juxtamembrane position immediately NH2-terminal to the transmembrane domain (Fig. 2, b, c, and d). However, the p35 was unique in that its NH2-terminal extracellular domain contained a stretch of 80 amino acid residues (78–157) that were ∼74% identical and ∼83% similar to a hypothetical human DNA MMR enzyme-related protein (the "PMSR6" gene; Figs. 1 c and 2 d; Horii et al., 1994; Nicolaides et al., 1995). RT-PCR using primers corresponding to the PMSR6- and UPIII-related regions yielded the expected p35 PCR products, thus proving the colinear existence of these two domains in bovine p35 (Fig. 2 a and unpublished data).

P35 expression is urothelial-specific and differentiation-dependent

Northern and PCR analyses showed that p35 expression was urothelium-specific in both bovine (Fig. 3, compare a with b
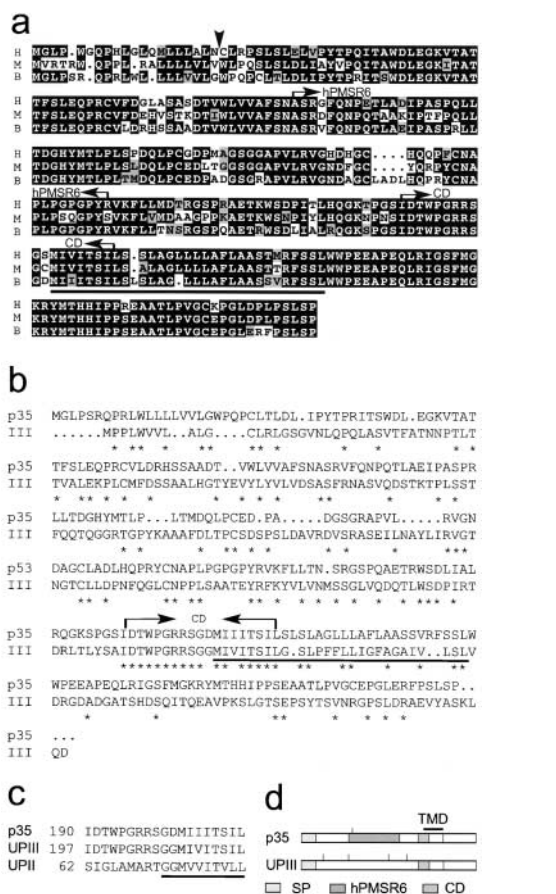
Figure 2.   **The amino acid sequence of p35 is conserved and is highly homologous to uroplakin III.** (a) cDNA-deduced amino acid sequences of p35 from human (H), mouse (M), and bovine (B). Arrowhead marks the end of signal peptide. HPMSR6 and CD denote the hPMSR6 homologous domain, and the conserved 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.

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
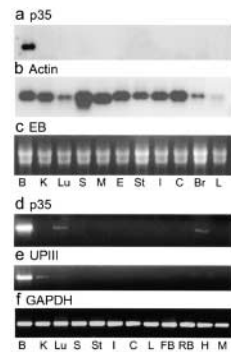

Figure 3.   **Urothelial specificity of p35 in bovine and mouse.** For bovine tissues (a–c), 20 μg total RNA from 11 tissues was separated on a formaldehyde gel, transferred to a nylon membrane, and probed with (a) p35 cDNA, (b) β actin cDNA, (c) an ethidium bromide (EB)–stained gel. For mouse tissues, 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).
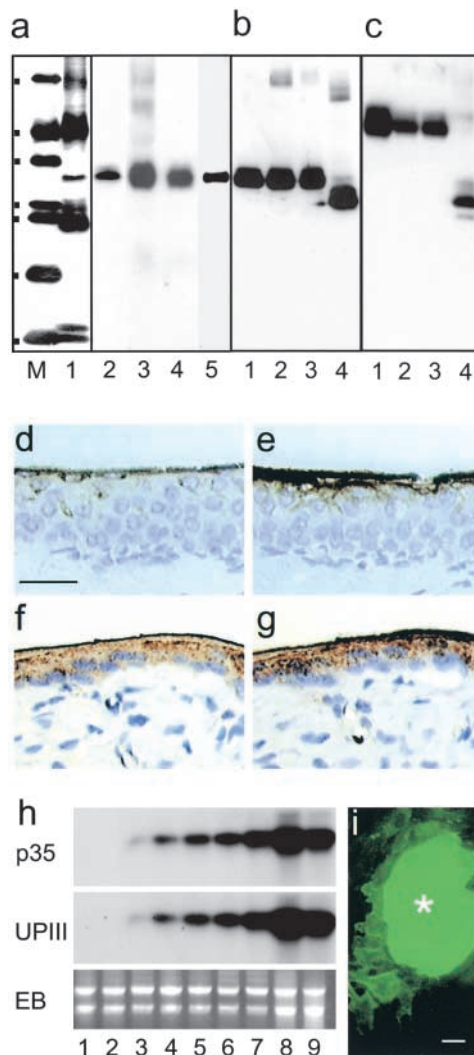

Figure 4.   **The urothelial differentiation-dependent expression of p35.** (a) A rabbit antiserum was generated to a synthetic peptide (positions 268–279 in the cDNA-deduced protein sequence of p35) and used to immunoblot total bovine urothelial proteins (lane 2), total membrane proteins (lane 3), purified 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

band in purified bovine (Fig. 4 a, lane 4) and mouse (Fig. 4 a, lane 5) urothelial plaques. Immunoblot data showed that p35 and UPIII shared many properties: they have a 30-kD core protein (Fig. 4, b and c); they were associated with the superficial umbrella cells of bovine urothelium (Fig. 4, d and e) and with the suprabasal cells of mouse urothelium (Fig. 4, f and g; Lin et al., 1995); and they were expressed in a differentiation-dependent manner in cultured bovine urothelial cells (Fig. 4, h and i). Finally, we found that p35 was enriched >100-fold in purified bovine urothelial plaques in comparison with the total bovine urothelial protein extracts (Fig. 4 a). These results indicated that p35 expression was urothelial-specific and differentiation-dependent, and that p35 protein, like uroplakin III, was urothelial plaque-associated (Figs. 1 a and 4 a).

Heterodimer formation between P35 and uroplakin Ib

Our recent transfection experiments supported the concept that the four major uroplakins form two pairs consisting of UPIa–UPII and UPIb–UPIII (Wu et al., 1995; Tu et al., 2002). For example, we found that UPIII expressed alone in 293T cells was trapped in the ER; however, cotransfection of UPIII with its putative partner UPIb (but not UPIa) resulted in its release from the ER, allowing it to reach the cell surface (Tu et al., 2002). This finding indicated that UPIII was a partner of UPIb, and raised the question whether the UPIII-related p35 might also partner with UPIb. To answer this question, we cotransfected 293T cells with cDNAs of p35 and each of the four uroplakins (Ia, Ib, II, and III). These experiments were conducted using 293T cells, which could be easily transfected and had no endogenous uroplakins, thus facilitating the studies of heterodimer formation. Immunoprecipitation using a p35 antibody established the coprecipitation of p35 with UPIb, but not with other uroplakins (Fig. 5 a). In another experiment, we assessed the glycosylation status of p35 in singly (p35 alone) and doubly (p35 plus one uroplakin) transfected 293T cells. We found that p35, when expressed alone, was synthesized as a 32-kD protein that was Endo H sensitive, indicating that it harbored high mannose-type sugars (Fig. 5 b). Cotransfection with UPIb, but not with other uroplakins, rendered Endo H resistance to p35, confirming the ability of the p35–UPIb complex to exit from the ER. Consistent with this interpretation, our metabolic labeling experiment using double-transfected 293T cells showed that p35 and uroplakin Ib could be coprecipitated (by anti-p35 antibody) immediately after a 10-min pulse labeling before p35 acquired Endo H resistance, indicating that the formation of p35/UPIb heterodimer occurred in the ER (Fig. 5, c and d). Finally, immunofluorescent staining of transfected 293T cells showed that whereas singly expressed p35 was ER-associated (Fig. 6 a, right) with no cell surface staining (Fig. 6 a, left), p35 expressed in the presence of uroplakin Ib was exposed on the surface of living cells (Fig. 6 b and data not depicted).

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), or with p35 plus uroplakin Ib cDNAs (lanes 4–6). Total cellular proteins were isolated 24 h later, and immunoblotted using antibodies to p35 (top) or uroplakin Ib (bottom). The samples were either 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 [^{35}S]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 UPIIIb cDNAs (as in panel c) were either treated with endoglycosidase H (+) or incubated

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Fig. 4 legend continued from previous page

and 18s ribosome RNA as a loading control. Note the differentiation-dependent expression of both p35 and UPIII. (i) 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.
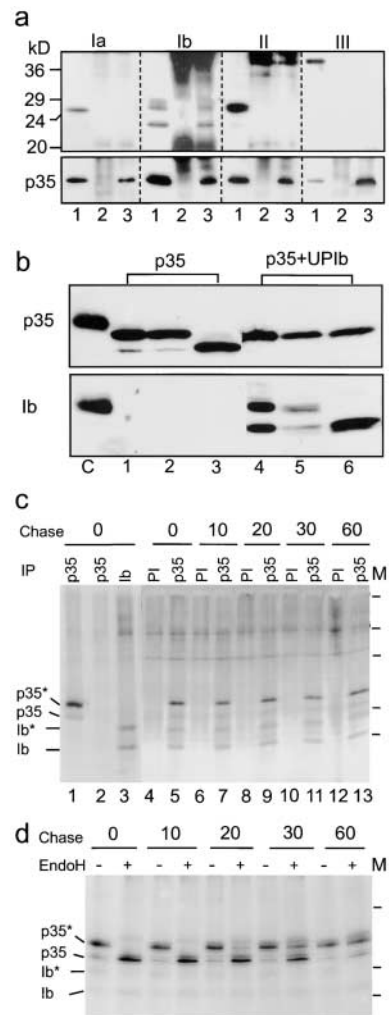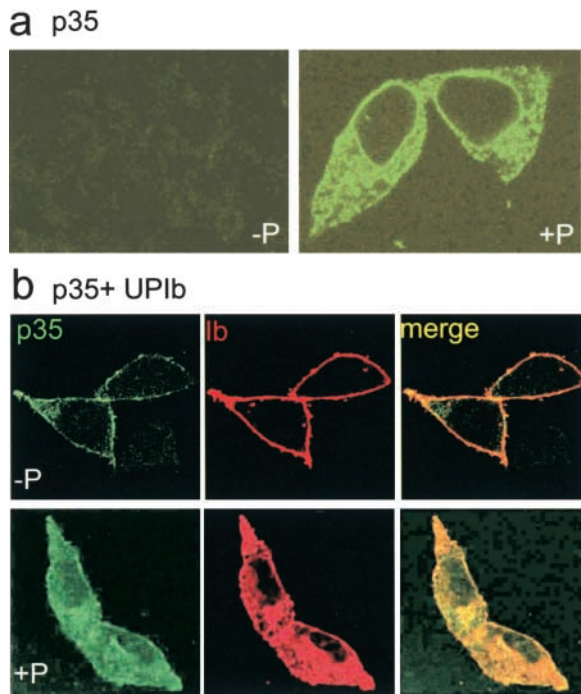
a p35

b p35 + UPIb

Figure 6. Uroplakin Ib enables p35 to reach the cell surface.
(a) 293T cells transfected with p35 cDNA alone were immunostained without (−P) or with (+P) saponin permeabilization. Note the inability of p35 to reach the cell surface. (b) 293T cells were cotransfected with p35 and HA-tagged UPIb cDNAs, and immuno-stained using antibodies to p35 (green) and to the HA tag of UPIb (red), without (−P) or with saponin permeabilization (+P). Note the colocalization of p35 and UPIb in permeabilized cells, and the ability of p35 to reach the cell surface in the presence of UPIb.

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 up-regulation 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/UPIb pair, both mRNAs increased approximately sixfold (Fig. 7 a), whereas the pro-

overnight in a buffer without Endo H enzyme (−) as controls. M denotes molecular mass markers (from top to bottom: 37, 25, and 20 kD). Note that the newly synthesized p35 were Endo H sensitive, but acquired Endo H resistance after 20–30 min when they entered into the Golgi. Although the amount of UPIb was a bit low in this experiment, this uroplakin never acquired Endo H resistance consistent with our earlier data (Tu et al., 2002).
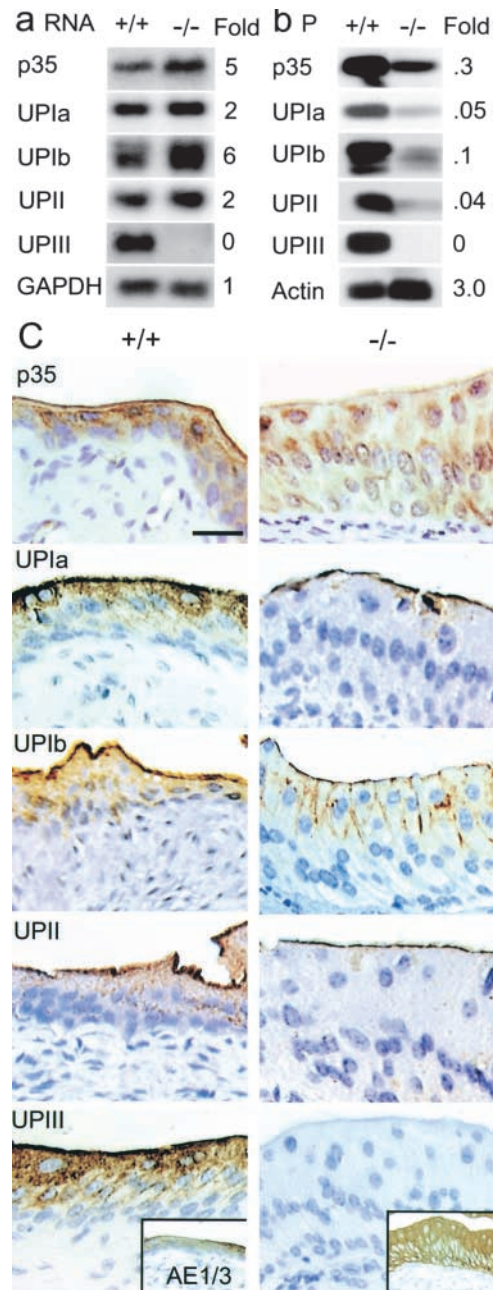

Figure 7. Perturbed p35 expression in UPIII deficiency mice.
(a) Northern analysis. Total RNAs from wild-type (+/+) and UPIII knockout (−/−) mouse bladders were resolved electrophoretically and probed with cDNA of mouse p35, uroplakins Ia, Ib, II, III, and GAPDH. Densitometry showed a five- to sixfold increase of p35 and UPIb messages in the −/− mice bladder, and an approximately twofold increase of UPIa and UPII messages. (b) Immunoblot analysis. Total urothelial proteins (20 μg) from normal and uroplakin III–deficient mice were immunoblotted. Note that uroplakins Ia, Ib, and II were reduced 10–25 fold in the UPIII-deficient mouse bladder, whereas the p35 level was reduced by only threefold. (c) Immunohistochemical analysis. Paraffin sections of normal and UPIII-deficient bladders were immunostained with antibodies against p35, uroplakins Ia, Ib, II, III, and keratins (a mixture of AE1 and AE3 antibodies). Note the reduced expression of p35 and uroplakins Ia, Ib, and II in the hyperplastic uroplakin III-deficient urothelium. Also note the abnormal targeting of p35 (cytoplasmic accumulation) and UPIb (to basal lateral surface). Bar, 50 μm.

teins 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 mistargeted 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 located 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
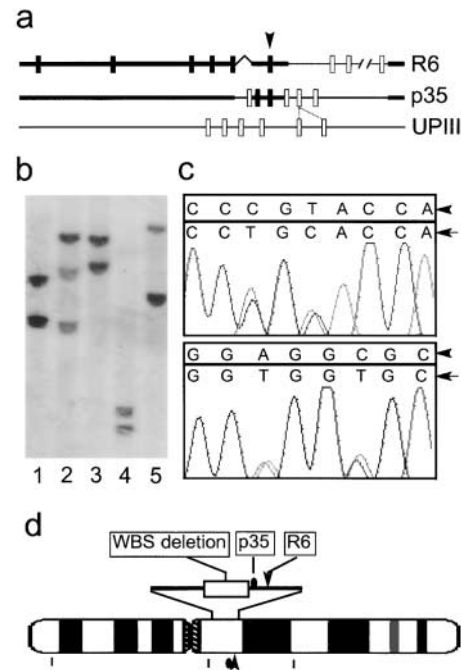

Figure 8. **Genomic structure of the human p35 gene.** (a) The organization of human R6, p35, and uroplakin III genes. Note the extensive sequence homologies between p35 and R6 genes (thick bars) suggesting gene duplication. Arrowhead denotes an exon encoding the 80 amino acids shared by p35 and R6 proteins. (b) Southern blot. Human genomic DNA was digested with various restriction enzymes including BamHI (lane 1), EcoRI (lane 2), HindIII (lane 3), PstI (lane 4), and XbaI (lane 5). The DNA fragments were separated electrophoretically and hybridized with a partial human p35 cDNA (covering exons 2 and 3, a sequence shared by p35 and R6 genes). Note that at least two p35-related genomic fragments were detected in all digestions consistent with the existence of two independent genes, i.e., p35 and R6. (c) Two pairs of primers were used to amplify exons 2 and 3 of human p35 gene (an area highly homologous to intron 5 and exon 6 of the R6 gene), and the PCR products were sequenced. The two PCR products indeed contained the expected p35 (arrowhead) and R6 (arrow) genomic sequences (p35 sequence according to human cosmid RP11-340a14, and hPMSR6 to cosmid RP11-506L7), thus confirming the existence of the two highly related genes. (d) Location of human p35 and hPMSR6 genes on human chromosome 7 and their relation to the deletion region of the Williams-Beuren syndrome (open box). Dot, arrowhead, and vertical bars denote p35 gene, R6 gene, and other R6-related genomic loci, respectively.

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
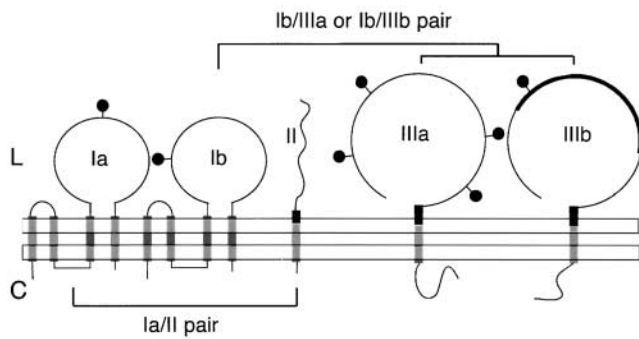
Figure 9. **Specificity of uroplakin heterodimer formation.** A schematic diagram showing the specific interactions between uroplakins Ia and II, and uroplakin Ib with either IIIa or IIIb (p35). The gray and black thick bars denote the transmembrane domains and the 19-amino acid CD that is shared by UPIIIa and UPIIIb, respectively. The NH$_2$-terminal 12 of the CD is also shared by UPII. Solid circles denote sugar moieties. L and C denote luminal and cytoplasmic side, respectively.

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 UPIIIa 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 UPIb converts the N-linked oligosaccharides of UPIIIb from an Endo H–sensitive to an Endo H–resistant form (Fig. 5 b), indicating that UPIb 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 coprecipitate 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 UPIIIa 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. 1a 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 UPIIIa 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 UPIb. 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; Harfe and Jinks-Robertson, 2000). Defects in hPMS2 have been shown to play a role in hereditary nonpolyposis colon cancer (Nicolaides et al., 1994; Hamilton et al., 1995). Moreover, ablation of mouse PMS2 gene leads to the formation of sarcomas, lymphomas, and intestinal adenomatous polyp (Baker et al., 1998). PCR amplification led to the identification of >17 human PMS2-related cDNAs, and hPMSR6 is one of them (Horii et al., 1994; Nicolaides et al., 1995). All these PMS2-related genes are mapped to chromosome 7, are homologous with the 5′ portion of PMS2, and contain several motifs highly conserved among various DNA MMR enzymes, such as KELVEN and GFRGEAL (Prolla et al., 1994), suggesting possible enzymatic domains. However, no data are as yet available about the expression of hPMSR6 in terms of its tissue distribution and protein function.

UPIIIb gene is telomeric of the frequently deleted region of the WBS

WBS is a neurodevelopmental disorder affecting multiple organs with distinctive facial features, congenital cardiovascular anomalies, mental retardation, growth deficiency, and occasional infantile hypercalcemia. Importantly, WBS patients have an increased prevalence of urinary symptoms and voiding dysfunctions (Babbitt et al., 1979; Morris et al., 1990; Blane et al., 1994; Perez Jurado et al., 1996). For example, Schulman et al. (1996) reported that ~10% of WBS patients had bladder diverticula and uninhibited detrusor

contraction. Pankau et al. (1996) reported that 17.7% of the WBS patients had bladder diverticula and renal abnormalities. Other genitourinary manifestations include urethral stenosis, vesicoureteral reflux, and recurrent urinary tract infection (Lashkari et al., 1999). Stoermer et al. (1984) reported that 12 out of 14 of children with all signs of WBS had renal and lower urinary tract abnormalities, but only 1 out of 5 of children with only typical cardiovascular findings (without pathognomonic faces or major mental retardation) showed urinary tract problems. WBS is caused by the heterozygous deletion of parts of chromosome subband 7q11.23 amounting to 1.5 to 2 Mb (Francke, 1999; Hockenhull et al., 1999; Peoples et al., 2000). Such deletions occur due to the presence of long stretches of duplicated sequences flanking this subband, thus facilitating inter- or intra-chromosomal crossover events (Dutly and Schinzel, 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 HybriZAP 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 λ 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 Sciex) as described previously (Resing 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 ^{32}P-labeled probes. For RT-PCR, cDNA was synthesized using a SuperScript™ first-strand synthesis system (GIBCO BRL). Primers for bovine p35 were forward, 5′-CGTGCTTAGGGTGGGCAATG-3′, and reverse, 5′-ATCATGTCTCCACTCCGCCG-3′. Primers for mouse p35 were forward, 5′-ACCTGGAAGGGAAGATCAC-3′, and reverse, 5′-AACGTTTTCCCATGAAGGAG-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) VLDRHSSAADTVW, (2) TNSRGSPQAETRWSD, and (3) EPGLERFPSLSP 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 NH$_2$ 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 Ultralink Iodoacetyl column (Pierce Chemical Co.). The eluted antibodies were concentrated using Centricon-30 (Amicon).

Immunoblotting and enzymatic deglycosylation
Proteins were separated by SDS-PAGE on 17% 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 by the enhanced chemiluminescence Western blotting detecting system (Pierce Chemical Co.) and exposed to Fuji x-ray film at RT. For enzymatic deglycosylation, bovine urothelial plaques 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 DME containing 15% FCS, 0.5 µg/ml hydrocortisone, 5 ng/ml cholera toxin, 5 µg/ml insulin, and 15 ng/ml 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 reached ∼70% confluence. Urothelium were collected at different stages of differentiation and 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 an mAb against UPIII (Liang et al., 2001; Riedel et al., 2001). Primary antibodies were detected with specific HRP-conjugated secondary antibodies (RCN). 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 Axiophot 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 UPIb cDNA. 293T cells were maintained in DME supplemented with 10% FBS. The FuGENE6 reagent (Roche) was used for transient transfection. 18 h before transfection, 293T cells were plated in 6-well plates (6 × 10⁵/well). cDNA was mixed with FuGENE6 (Roche; 1:3, wt/vol) in serum-free DME, 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 deoxylcholate, 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 DME containing 5% dialyzed bovine serum. The cells were then pulse-labeled for 10 min with [³⁵S]methionine (ICN Biomedicals) at 0.05 mCi/ml. Chase was done at 37°C for the indicated periods with DME 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 antibody-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 UPIb (1:200), 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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