Roles of uroplakins in plaque formation, umbrella cell enlargement, and urinary tract diseases

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The apical surface of mouse urothelium is covered by two-dimensional crystals (plaques) of uroplakin (UP) particles. To study uroplakin function, we ablated the mouse UPII gene. A comparison of the phenotypes of UPII- and UPIII-deficient mice yielded new insights into the mechanism of plaque formation and some fundamental features of urothelial differentiation. Although UPIII knockout yielded small plaques, UPII knockout abolished plaque formation, indicating that both uroplakin heterodimers (UPIa/II and UPIb/III or IIIb) are required for plaque assembly. Both knockouts had elevated UP1b gene expression, suggesting that this is a general response to defective plaque assembly. Both knockouts also had small superficial cells, suggesting that continued fusion of uroplakin-delivering vesicles with the apical surface may contribute to umbrella cell enlargement. Both knockouts experienced vesicoureteral reflux, hydronephrosis, renal dysfunction, and, in the offspring of some breeding pairs, renal failure and neonatal death. These results highlight the functional importance of uroplakins and establish uroplakin defects as a possible cause of major urinary tract anomalies and death.

Introduction

The apical surface of mammalian urothelium that is in contact with the urine is highly specialized, featuring two-dimensional (2D) crystals (urothelial plaques) of hexagonally packed 16-nm protein particles (Hicks and Ketterer, 1969; Vergara et al., 1972; Staehelin et al., 1972; Brisson and Wade, 1983; Walz et al., 1995; Kachar et al., 1999; Oostergetel et al., 2001; Min et al., 2003). These plaques contain four major uroplakins (UPs): Ia (27 kD), Ib (28 kD), II (15 kD), and III (47 kD) (Wu et al., 1993). Within the plaques, these four major uroplakins are organized into two heterodimers consisting of Ia/II and Ib/III, as demonstrated by chemical cross-linking (Wu et al., 1995) and protein isolation (Liang et al., 2001). In addition, transfection studies show that when individual uroplakins are expressed in 293T cells, they are retained in the ER; however, coexpression of uroplakins Ia plus II or Ib plus III permits the heterodimers to exit from the ER; these events suggest that the formation of specific heterodimers is a prerequisite for uroplakins to reach the cell surface (Tu et al., 2002).

To study the functional and disease implications of uroplakins, we previously generated uroplakin III–deficient mice using the gene-targeting approach (Hu et al., 2000). Such mice lack a typical urothelial umbrella cell layer. They have a reduced urothelial plaque size, compromised urothelial permeability barrier function, and retrograde flow of urine from the bladder into the ureters (vesicoureteral reflux [VUR]). In ∼70% of the mice, accumulation of urine in the renal pelvis (hydronephrosis) occurs (Hu et al., 2000, 2002). These results establish that uroplakin III is an integral subunit of urothelial plaques, which contribute to the permeability barrier function.
of the urothelial apical surface, and suggest that urothelial defects may play a role in VUR (Hu et al., 2000, 2002).

Some of the phenotypes of these UPIII-deficient mice remain hardly understood. For example, the observation that UPIII deficiency leads to the formation of small plaques seems incompatible with our current assumption that all four major uroplakins are required for the formation of the 16-nm particle (Sun et al., 1999). One possible explanation is that UPIIIb, a newly discovered, minor UPIII isoform (Deng et al., 2002), is responsible for the formation of the residual plaques observed in the UPIII-deficient urothelium. If it is, one would predict that knockout of uroplakin II, which does not have a known isoform, should completely abolish plaque formation. Another question has to do with the possible role of uroplakin mutation in VUR. Although knockout of the UPIII gene leads to VUR in mice, screening of human VUR patients revealed that polymorphism of uroplakin genes is only marginally associated with VUR (Giltay et al., 2004; Jiang et al., 2004). Moreover, although polymorphism was found in these VUR patients, no deletion, truncation, or frameshift mutations have been detected so far (Giltay et al., 2004; Jiang et al., 2004). The role of uroplakin defects in VUR and other lower urinary tract anomalies thus remains unclear.

To address these issues, we have genetically ablated the mouse UPII gene to perturb the other uroplakin pair consisting of UPIa/II. This led to the entrapment of UPIa in the ER, but allowed the remaining UPIb/III pair to reach the apical urothelial surface. No 16-nm particle or apical plaques were formed, however, which indicates that both uroplakin heterodimers were required for plaque formation. The small size of the superficial cells in both UPIII- and UPIII-deficient urothelia suggested that the incorporation of fully assembled uroplakin plaques into the apical surface through fusion of the fusiform vesicles with the apical membrane played a role in the enlargement of umbrella cells. Because entire litters of UPIII-deficient mice from some breeding pairs (originating from the inbred 129/SvEv and outbred Swiss Webster strains) reproducibly died 8–10 d postnatally, as a result of renal failure, it seems that major uroplakin defects such as gene deletion, frameshift, or truncation might cause urinary tract anomalies that, in severe cases, could lead to death. These results highlight the critical functional importance of uroplakins in the formation of a specialized urothelial apical surface, and shed light on the mechanisms of plaque formation, umbrella cell enlargement, and certain urinary tract abnormalities.

**Results**

**Inactivation of the mouse uroplakin II gene**

After transfecting embryonic stem (ES) cells (from 129/SvEv mice) with a vector designed to delete the first four exons and a part of the fifth exon of the mouse uroplakin II gene (Fig. 1 A), we screened 320 ES cell colonies and found 4 that were harboring the correct homologous recombination events as determined by long template PCR (Fig. 1 B) and Southern blot (not depicted). 18 chimeric mice from three ES cell lines were generated. After breeding to black Swiss Webster mice, the inheritance of the unique uroplakin II–deficient allele was confirmed by PCR analysis of the genomic DNA using a mixture of two pairs of primers that generate two PCR products characteristic of the wild-type and KO alleles, respectively; Neo, neomycin-resistant gene; TK, thymidine kinase of the herpes simplex virus; B, BamHI; C, ClaI; E, EcoRI; S, SacI; X, Xhol. (B) PCR analysis of the genomic DNA using a mixture of two pairs of primers that generated 4.0-kb and 3.8-kb PCR products characteristic of the wild-type (+) and KO (−) alleles, respectively. M, +C, and −C denote size markers, positive controls (+/− genomic DNA or vector used as templates), and a negative control (no template), respectively. (C) Northern blot analysis. 5 μg of total RNA samples was resolved electrophoretically and probed with cDNA of mouse uroplakins Ia, Ib, II, and III, and glyceraldehyde phosphate dehydrogenase (GAPDH) as a loading control. Note, in the −/− animals, the absence of UPII message and the absence of UPIb message, the significant increases in UPIa and UPIIIb messages in C serving as a loading control. (E) Immunoblot analysis of mouse urothelial proteins Ia, Ib, II, and III, and glyceraldehyde phosphate dehydrogenase (GAPDH) as a loading control. Note, in the −/− mice the absence of UPII protein, the significant decrease in the levels of the other three uroplakin proteins, and the absence of the highest mol wt (−30 kD) UPIIIb species due to defective glycosylation.

![Figure 1](image-url)
protein levels of uroplakin Ia, UPII’s partner, and of uroplakins Ib and III (of the uroplakin Ib/III pair) were reduced 10–20-fold (on a per total cellular protein basis) when compared with those of the normal control mice (Fig. 1 E). The mRNA levels of UPIa and UPIII were slightly up-regulated (about twofold), whereas that of UPIb was drastically up-regulated by 10–20-fold (Fig. 1 C; see Discussion).

Uroplakin expression and plaque formation
Consistent with the UPIII knockout results, the UPII-deficient urothelium lacked a typical superficial umbrella cell layer (Fig. 2 b). This urothelium was hyperplastic, as indicated by an elevated level of BrdU incorporation (the labeling index increased almost 100-fold from 0.12%, which is normal, to 10.7%; Fig. 2, c and d), and was three- to sixfold thicker than normal (Fig. 2, a and b). Immunohistochemical staining with monospecific rabbit and mouse antibodies to individual uroplakins confirmed that all four major uroplakins were preferentially expressed in superficial umbrella cells in normal mouse urothelium (Fig. 3, a, c, e, g, and i; Hu et al., 2000). The staining of the UPII-deficient urothelium showed that UPIa (the partner of UPII) was diffusely distributed in all upper cells, which is consistent with its entrapment in the ER (Fig. 3 d). Uroplakins Ib and III (of the other uroplakin pair), although expressed at a lower level than normal, were clearly associated with the apical, as well as some basal/lateral, cell surface(s) (Fig. 3, f and j).

Unlike normal urothelium, which was covered by large squamous superficial umbrella cells that could be as large as 100 μm in diameter (Fig. 4 a) and were covered by rigid-looking plaques (Figs. 4 c and 5 a), superficial cells of the UPII-deficient urothelium were uniformly small (20–30 μm; Fig. 4 b) and were completely devoid of the rigid-looking, apical
plaque-free apical surface. The urothelia from age-matched 3-mo-old wild-type (a and c) and UPII-deficient (b and d) mice were examined by scanning EM (a and b) and quick-freeze deep etch transmission EM (c and d). Note the replacement of large surface umbrella cells (U) by small superficial cells (S) (a and b), and the complete absence of the 16-nm particles and urothelial plaques on the apical surface of the UPII-deficient urothelium (d). cyt, cytoplasm; m, membrane; ups, uroplakin particles. Bars: (a and b) 20 μm; (c and d) 0.2 μm.

Figure 4. The UPII-deficient urothelium had small superficial cells and a particle-free apical surface. The urothelia from age-matched 3-mo-old wild-type (a and c) and UPII-deficient (b and d) mice were examined by scanning EM (a and b) and quick-freeze deep etch transmission EM (c and d). Note the replacement of large surface umbrella cells (U) by small superficial cells (S) (a and b), and the complete absence of the 16-nm particles and urothelial plaques on the apical surface of the UPII-deficient urothelium (d). cyt, cytoplasm; m, membrane; ups, uroplakin particles. Bars: (a and b) 20 μm; (c and d) 0.2 μm.

Figure 5. The replacement of the fusiform vesicles by small spherical vesicles that delivered the remaining uroplakin pair in uroplakin-deficient urothelium. The urothelia from age-matched 3-mo-old wild-type (a, c, and d), UPII-deficient (b, g, and h), and UPIII-deficient (e and f) mice were examined by thin section (a and b) and immunolabeling (c–h) transmission EM. Normal uroplakin-delivering fusiform vesicles (a, c, and d) were completely replaced by numerous small, spherical, immature-looking spherical vesicles in UPIII-deficient (e and f) and UPI-deficient (b, g, and h) urothelia. Note the strong labeling of normal fusiform vesicles (*) by antibodies to uroplakins Ia (c) and Ib (d), the moderate staining of the spherical vesicles in the UPIII-deficient urothelium (which still expressed the UPIIIb isoform; e and f), and the weak staining of the small vesicles (arrows) in the UPII-deficient urothelium by anti-UPIII (g) and by a rabbit antiserum to total uroplakins (h). Arrowheads (b) indicate the smooth apical surface of the UPIII-deficient urothelium. Fv, fusiform vesicle; P, plaque; Sv, small vesicle. Bars: (a and b) 0.5 μm; (c–h) 1 μm.

VUR, ureteral obstruction, and hydronephrosis

By administering an India ink solution into the bladders of live, anesthetized mice (Fig. 6, a and b), we determined the hydrostatic pressures at which micturition (Pm) and VUR (Pv) occurred. Normal mice had a Pm of 28 cm of H2O pressure (Fig. 6 c), and most of them did not reflux—unless the urethra was ligated, causing outlet obstruction, and the hydrostatic pressure was increased to 40–80 cm of H2O (Fig. 6 d, WT). Although the UPII knockout mice had a normal micturition pressure of ∼24 cm of H2O (Fig. 6 c), >50% (11 out of 20) of the mice refluxed at a pressure lower than this (Fig. 6, d and e). Many of the (∼1/4) mice developed severe hydronephrosis with a greatly expanded renal pelvis (Fig. 7, b and d) and associated renal morphological changes (Fig. 7, a–f). To determine whether reflux caused hydronephrosis, we calculated the difference between the Pm and the Pv of each ureter (Pv [hydrostatic pressure to the kidney] = Pm – Pr). For a normal mouse that had a Pm of 25 cm of H2O and a Pr of 50 cm, an intravesicular pressure of >25 cm of H2O would result in micturition and dissipation of pressure. However, for a UPII knockout mouse that had a Pm of 25 cm of H2O but a lower Pr of 18 cm of H2O, intravesicular pressures of 18–25 cm of H2O would lead to reflux, thus potentially transmitting up to 7 cm of hydrostatic pressure to the kidney (Pv). We wanted to see whether this renal pressure correlated with the grade (G) of hydronephrosis—which we defined as G = D/T, where D was the internal diameter of the renal pelvis and T was the thickness of the remaining renal parenchyma (Fig. 7, c and d). If reflux were a main cause of hydronephrosis, there should have been a positive correlation between Pr and G. However, we found that these two parameters were independent (P > 0.2), which sug-
suggests that reflux could not be the major etiology of hydronephrosis in this system (Fig. 7 g).

Another possible cause of hydronephrosis is ureteral obstruction, which impedes the flow of urine from the kidney. To investigate this possibility, we performed i.v. pyelogram (IVP) by injecting 3 μl/g (of body weight) of Omnipaque into the orificial sinus, followed by serial radiography (Fig. 8, a and b). The results indicated that UPII knockout mice had a significantly delayed excretion of Omnipaque, indicating obstruction. Serial sectioning of the urinary tracts of UPII knockout mice revealed areas of the ureter with epithelial polyps or complete epithelial occlusion, which caused structural obstruction (Fig. 8, c–h; see Discussion). These results suggest that both VUR and structural and/or functional obstruction of the ureters may be responsible for the observed hydronephrosis in UPII knockout mice.

Renal dysfunction and death

Using a filter paper assay, we found that UPII knockout mice had higher volume per micturition (Fig. 9 b), micturition frequency (Fig. 9 c), and total urine output (Fig. 9 d) than normal mice; no statistically significant gender differences were noted (Fig. 9, b–d). The levels of many urinary components, including uric acid, creatinine, potassium, sodium, and chloride ions, were slightly reduced (Fig. 9, f–j), possibly because of defects in mechanisms of urine concentration (Fig. 9 d). The concentration of blood urea nitrogen (BUN) almost doubled (Fig. 9 e), suggesting a compromised renal function. Interestingly, although most of the breeding pairs yielded litters that survived into adulthood, the litters of some breeding pairs (129/SvEv × Swiss Webster) reproducibly died around days 8–10 postnatally (Fig. 10, a and b). These litters were characterized by retarded growth (Fig. 10 c) and a sharp surge in the BUN level (Fig. 10 d), which suggests renal failure as a cause of death, possibly resulting from ureteral obstruction (Fig. 8, c–h) and a defective renal pelvis urothelium that normally expressed uroplakins (Fig. 10 e).

Discussion

Mammalian urothelium has three unique biological features: its apical membrane is highly specialized, harboring 2D crystals of hexagonally packed 16-nm particles (Hicks and Ketterer, 1969; Vergara et al., 1969; Staehelin et al., 1972; Brisson and Wade, 1983); its superficial umbrella cells are greatly flattened and expanded, with a diameter reaching over 100 μm (Porter and Bonneville, 1963; Hicks, 1965; Koss, 1969; Lewis, 2000; Veranic et al., 2004; for review see Hicks, 1975); and it is one of the slowest-cycling stratified epithelia, with a BrdU-labeling index of <0.1% (Martin, 1972; Farsund, 1975). The ablation of a single gene that encodes uroplakin II perturbed all of these urothelial properties and led to major urinary tract abnormalities.

Mechanism of uroplakin plaque formation

As mentioned earlier, the four major uroplakins can form two heterodimer pairs consisting of uroplakins Ia/II and Ib/III (Wu et al., 1995; Liang et al., 2001; Tu et al., 2002). Transfection
studies indicated that the formation of correct heterodimer is required for uroplakins (except UPIb; see below) to exit from the ER and to reach the cell surface (Tu et al., 2002). Because 293T (a human embryonic kidney–derived cell line) cells were used in these transfection studies, the physiological significance of in vitro data obtained using nonurothelial cells might be questioned. Our in vivo data show that although UPIa is distributed throughout the cytoplasm of the UPII-deficient urothelium, suggesting ER entrapment filling the urothelial lumen (f), sometimes forming a complete occlusion (h). B, bladder; EO, epithelial occlusion; EP, epithelial plug; K, kidney; l, lumen. Bar, 50 μm.

Although cultured bovine urothelial cells continue to synthesize large amounts of all the uroplakins, these cells do not form any mature-looking, uroplakin-delivering fusiform vesicles that are characteristic of the in vivo urothelial cells, and their apical cell surface is completely devoid of urothelial plaques (Surya et al., 1990; Chen et al., 2003). Based on what we have learned from cultured keratinocytes (Weiss et al., 1984; Schermer et al., 1989), cultured urothelial cells most likely mimic in vivo regenerating urothelium (Surya et al., 1990; Chen et al., 2003). The inability of regenerating urothelial cells to assemble plaques could be functionally important, because a cell surface laden with the rigid-looking plaques may be incompatible with cell migration and proliferation during wound repair. Therefore, although we can study certain early steps of uroplakin interaction in the currently available, cultured urothelial cells and transfected 293T cells, the final stages of uroplakin assembly and 2D crystal formation need to be studied using the in vivo urothelium. Our present finding that the UPII–deficient urothelium lacks 16-nm particles (Fig. 3, f and d) indicates that, although the UPIb/III pair can be delivered to the apical surface (Fig. 3, f and j) using the small vesicles (~150–200 nm in diameter) accumulated in the UPII-deficient superficial cells (Surya et al., 1990; Chen et al., 2003), the UPIb/III pair by itself cannot form the 16-nm particle, let alone the urothelial plaques (Fig. 4 d). Severs and Hicks (1979) have shown that, in normal urothelium, 16-nm particles can be detected in early vesicles that have just budded off the Golgi apparatus, which suggests that the two uroplakin heterodimers (Ia/II and Ib/III) interact before they leave the Golgi apparatus to form the 16-nm particle. Together, these data suggest that uroplakins first form, in the ER, heterodimers (Tu et al., 2002), and possibly heterotetramers; the heterotetramers then assemble into the 16-nm particles in the Golgi apparatus or the trans-Golgi network, and these particles, once reaching a sufficient density in the post-Golgi vesicles, can then aggregate to form small and, later, large 2D crystals.

Inactivation of the UPII gene caused renal malfunction. (a) The standard curve of a filter paper assay for measuring the urine volume. (b) Volume per micturition. (c) Frequency of micturition. (d) Cumulative total micturition volume. (e) Blood urea nitrogen (BUN). Note the twofold increase of BUN in UPII-deficient mice (P = 0.00001) compared with wild-type mice. f–j show the urinary concentrations of uric acid (f), creatinine (g), potassium (h), sodium (i), and chloride (j). Urine and sera from 12 wild-type and 12 knockout 3-mo-old mice were pooled for these analyses. Error bars represent 1 SD.

Inactivation of the UPII gene caused ureteral obstruction. I.v. pyelogram of normal (a) and UPII knockout mice (b) established delayed excretion of contrast in the latter. c–h show the histological sections of the ureters of normal (c and e), UPII knockout (d and f), and UPII knockout (g and h) mice. Samples were taken from proximal (c and d) or distal (e–h) ureter. Note the formation of epithelial outgrowth filling the ureteral lumen (f), sometimes forming a complete occlusion (h). B, bladder; EO, epithelial occlusion; EP, epithelial plug; K, kidney; l, lumen. Bar, 50 μm.
Given our current assumption that both uroplakin pairs are required for the formation of the 16-nm particle, it was baffling that the UPIII-deficient urothelium still made 2D crystals, albeit small ones, from the 16-nm uroplakin particles (Hu et al., 2000). However, we now know that highly purified urothelial plaques contain a minor isoform of UPIII, which we recently characterized (Deng et al., 2002). This 35-kD protein was named uroplakin IIIb because it is urothelium specific, it shares a similar transmembrane topology and significant sequence homologies with UPIII (also known as UPIIIa), and it forms a heterodimer with UPIb, UPIIIa’s partner (Deng et al., 2002). This newly found uroplakin IIIb, which is up-regulated in UPIIa-deficient urothelium (Deng et al., 2002), should allow the formation of small amounts of heterotetramers containing heterodimers UPIb/UPIIIb and UPIa/UPIIIa. The heterodimers can then be delivered by the 150–200-nm vesicles (Fig. 5, e and f) and account for the formation of small surface plaques in the UP IIIa-deficient urothelium. This interpretation is consistent with our finding that the ablation of UPII, which does not have a known isoform, greatly diminishes the amounts of small vesicle-associated uroplakins and completely abolishes the formation of the 16-nm particles (Fig. 4, c and d).

We showed previously that the ablation of UP IIII led to abnormal synthesis and processing of UP Ib, i.e., the level of UP Ib mRNA was greatly increased, whereas the amount of UP Ib protein was reduced, became hypoglycosylated, and was mislocalized to the basal/lateral cell surface (Hu et al., 2000). Because UP II and UP Ib were known to interact, we speculated that these UP Ib changes were caused by the removal of its partner, UP IIII (Hu et al., 2000). Our finding that UP II ablation led to a similar up-regulation of the UP Ib mRNA level (Fig. 1 C) and hypoglycosylation of the UP Ib protein (Fig. 1 E) indicates, however, that these UP Ib changes represent a general response to a perturbed uroplakin assembly. This may explain why, in cultured human urothelial cells, the level of mRNA for UP Ib is greatly elevated compared with those for other uroplakins (Varley et al., 2004). Uroplakin Ib is also unique in that it is the only uroplakin that, when expressed alone in 293T cells, can exit from the ER to reach the cell surface (although it is required for its partner, UP IIII, to exit from the ER [Tu et al., 2002]); and in that it is the only uroplakin that is expressed in nonurothelial tissues, including corneal and conjunctival epithelia (Adachi et al., 2000) and, possibly, lung epithelium (Kallin et al., 1991; Olsburgh et al., 2003). These data indicate that uroplakin Ib is unique among the uroplakins in its regulation and function.

**Uroplakin assembly and umbrella cell enlargement**

Normal mouse urothelium is covered by large umbrella cells that have an average diameter of >100 μm (Hicks, 1965; Koss, 1969). The greatly enlarged superficial cells can minimize the intercellular space, thus contributing to the permeability barrier function of the apical urothelial surface (Negrete et al., 1996; Zeidel, 1996; Lewis, 2000; Apodaca, 2004). The mechanism by which these large umbrella cells, which are frequently tetraploid or octoploid (Farsund, 1975), are formed is unclear, although both arrested cytokinesis (Farsund, 1976; Farsund and Dahl, 1978) and cell fusion (Martin and Wong, 1981) have been suggested. Our finding that the superficial cells of both UP II- and UP IIII-deficient urothelia failed to enlarge (Fig. 4, a and b; Hu et al., 2000) suggests that urothelial plaque formation may play a role in umbrella cell enlargement. It is possible that the continued insertion of fusiform vesicles into the apical surface not only expands (Porter and Bonneville, 1963; Hicks,
for this reason, inactivation of angiotensin II receptor (AT2) gene (Fig. 1 A). Three primers were used for genotyping: one forward (5'-gagggagtaagacacaagctcaggaagga-3') and two reverse (5'-ctctagctgcttcgaggtcagc-3' for detecting a 3.8 kb product of the neomycin selection cassette, and 5'-caagttctgctgcttcgaggtcagc-3' for a 4.0 kb product of the native UPIII gene; Fig. 1, A and B).

Morphological studies
Mouse urothelium was examined by scanning EM (model JSM-840; JEOL) and transmission EM (model 200CX; JEOL [Hu et al., 2000]). Quick-freeze deep etch was performed as described previously (Kachar et al., 1999). Cell proliferation was assessed based on the nuclear incorporation of BrdU (Sigma-Aldrich). 19-d-old mice received five intraperitoneal injections of BrdU (Sigma-Aldrich).

Materials and methods
Production of the UPIII knockout mice
Genomic clones of the mouse UPIII gene were isolated from a 129/Ola mouse P1 genomic library (Genome Systems). The targeting vector was designed to delete the first four exons, and a part of the fifth, of the UPIII gene (Fig. 1 A). Three primers were used for genotyping; one forward (5'-gagggagtaagacacaagctcaggaagga-3') and two reverse (5'-ctctagctgcttcgaggtcagc-3' for detecting a 3.8 kb product of the neomycin selection cassette, and 5'-caagttctgctgcttcgaggtcagc-3' for a 4.0 kb product of the native UPIII gene; Fig. 1, A and B).
Brdu (100 ng/kg of body weight, in PBS), 1.5 h apart, and were killed 2 h after the final injection. Various tissues, including esophagus and urinary bladder tissues, were removed, fixed in 10% formalin overnight, and processed for immunostaining with a horseradish peroxidase–conjugated anti-Brdu monoclonal antibody (CHEMICON International). Samples were visualized with a microscope (Axioskop; Carl Zeiss Microimaging, Inc.) with 10×/0.32 and 20×/0.60 (Plan-APOCHROMAT) or 40×/0.75 (Plan-NEOFluar) objective lenses. Images were captured with a digital camera (model DHC-K5000; Sony) at room temperature. The images were processed in size and contrast/brightness with Adobe Photoshop 6.0.

Isolation of detergent-insoluble proteins from mouse urothelium

Mouse urothelial cells were homogenized in buffer A (10 mM Hepes, pH 7.5, 1 mM EDTA, 1 mM EGTA, and 1 mM PMSF), loaded onto a 1.6-M sucrose cushion (in buffer A), and centrifuged at 16,000 rpm for 25 min at 4°C (SW41; Beckman Coulter). The crude membranes concentrated at the interface were washed, with buffer A, treated with 2% Sarcosyl in buffer A for 10 min at 25°C, and pelleted. The detergent-insoluble membranes, in which the urothelial plaques were highly enriched, were washed with buffer A before they were solubilized in SDS-PAGE sample buffer and analyzed by SDS-PAGE, followed by silver nitrate staining, or by immunoblotting (Wu et al., 1994; Liang et al., 1999; Zhou et al., 2001).

Determination of reflux pressure and hydronephrosis

The reflux pressure was measured as the hydrostatic pressure [i.e., cm of H2O] at which an India ink suspension in PBS backflowed from the bladder to the urinaray (Wu et al., 1994; Liang et al., 1999; Zhou et al., 2001). Determination of the intrarenal pressure and hydronephrosis was performed using a capillary tube and a water column connected to the renal pelvis through a flexible plastic tube. The reflux pressure was measured as the maximum pressure at which India ink could be aspirated through the capillary tube into the water column. The hydronephrosis was determined as the degree of hydronephrosis, expressed as a percentage of the diameter of the renal pelvis, using a digital camera (model DHC-K5000; Sony).

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