Organization of uroplakin subunits: transmembrane topology, pair formation and plaque composition

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The apical surfaces of urothelial cells are almost entirely covered with plaques consisting of crystalline, hexagonal arrays of 16 nm uroplakin particles. Although all four uroplakins, when SDS-denatured, can be digested by chymotrypsin, most uroplakin domains in native urothelial plaques are resistant to the enzyme, suggesting a tightly packed structure. The only exception is the C-terminal, cytoplasmic tail of UPIII (UPIII) which is highly susceptible to proteolysis, suggesting a loose configuration. When uroplakins are solubilized with 2% octylglucoside and fractionated with ion exchangers, UPIa and UPII were bound as a complex by a cation exchanger, whereas UPIb and UPIII were bound by an anion exchanger. This result is consistent with the fact that UPIa and UPIb are cross-linked to UPII and UPIII, respectively, and suggests that the four uroplakins form two pairs consisting of UPIa/II and UPIb/III. Immunogold labelling using a new mouse monoclonal antibody, AU1, revealed that UPIII is present in all urothelial plaques, indicating that the two uroplakin pairs are not segregated into two different types of urothelial plaque and that all plaques must have a similar uroplakin composition. Taken together, these results indicate that uroplakins form a tightly packed structure, that the four uroplakins interact specifically forming two pairs, and that both uroplakin pairs are required for normal urothelial plaque formation.

Key words: bladder epithelium, differentiation, tetraspanin, urothelium.

INTRODUCTION

Over 90% of the apical surface of mammalian urothelium is covered by rigid-looking plaques that are 0.2–0.5 μm in diameter [1,2]. These membrane plaques, also known as asymmetric unit membranes or AUMs, are highly specialized, since they consist of two-dimensional, hexagonal crystalline arrays of 16 nm protein particles [3–7]. As the major differentiation product of urothelium [8,9], these urothelial plaques are thought to play an important role in urothelial structure and function. The proposed roles include the physical stabilization of the urothelial apical surface, a contribution to the remarkable permeability barrier function of the urothelium, and the reversible adjustment of the apical cell-surface area of the bladder [2,9–16].

Purified urothelial plaques contain four major proteins, which are named uroplakins Ia (UPIa; 27 kDa), Ib (28 kDa), II (15 kDa) and III (47 kDa) based on their *in situ* association with urothelial plaques [17–19]. A model of the transmembrane topology of uroplakins has been proposed (Figure 1), based on the locations of their potential transmembrane domains and glycosylation sites, as well as the protease susceptibility of their subdomains when individual *in vitro*-transcribed and -translated uroplakins are incorporated into dog pancreatic microsomes [19,20]. According to this model, almost all the hydrophilic domains of the four uroplakins protrude extracellularly and are responsible for the formation of the 16 nm particle [19,20]. A notable exception is UPIII, which has a cytoplasmic tail consisting of $\approx$ 50 amino acid residues that may be involved in mediating the binding of the urothelial plaque to a cytoskeleton [21]. Additional data are needed, however, to test the validity of this model.

The four major uroplakin proteins can be divided into two structurally related groups. UPIa and UPIb form one group as they share about 30% amino acid residues, both possessing four transmembrane domains (TMDs) [19]. These two proteins belong to a family of integral membrane proteins (tetraspanins), many of which are leukocyte-differentiation or tumour-associated antigens [19]. UPII and UPIII form another group as they both have a single TMD, and share a stretch of $\approx$ 12 amino acid residues located adjacent to the TMD at the N-terminal, extracellular side of the protein [21,22]. Interestingly, the structurally related UPIa and UPIb are preferentially cross-linked to UPII and UPIII, respectively, suggesting the existence of two uroplakin pairs [23]. It is unclear, however, how these two uroplakin pairs, if they indeed exist, contribute to the formation of 16 nm particles, which are the basic building blocks of the urothelial plaque. It is possible that the two uroplakin pairs co-exist in each 16 nm particle, thus all the plaques would have the same uroplakin

Abbreviations used: AUM, asymmetric unit membrane; UP, uroplakin; TMD, transmembrane domain.

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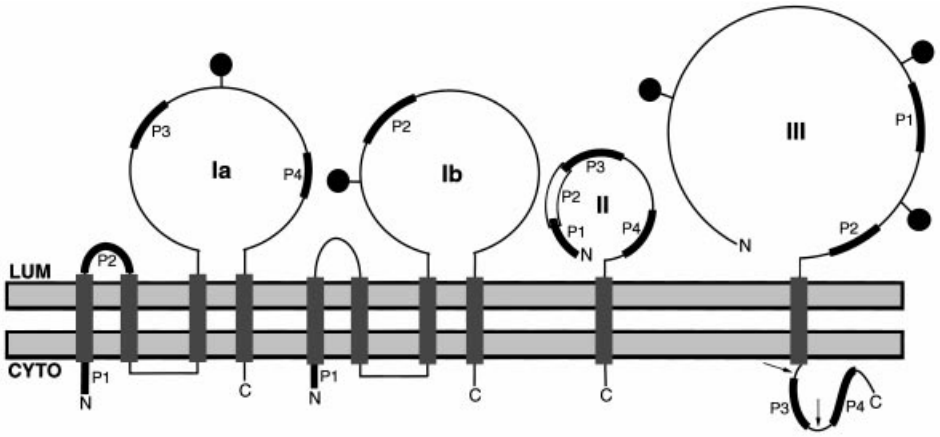

Figure 1 Hypothetical transmembrane topology of uroplakins and location of synthetic peptides

Potential transmembrane domains are represented by thick, vertical bars, and the luminal and cytoplasm leaflets of the lipid bilayer are labelled LUM and CYTO, respectively. The epitopes, defined by synthetic peptides, are represented by thick bars labelled P followed by a number (see Table 1 for sequences). Other abbreviations/symbols: N, N-terminus; C, C-terminus; 'lollipop', sugar moiety. Arrows denote two accessible chymotrypsin digestion sites on the cytoplasmic tail of UPIII (see Figure 2).

composition. Alternatively, the two uroplakin pairs may form separate particles that form different plaques, thus raising the possibility of heterogeneity in plaque composition.

The concept of uroplakin pairs has been based solely on the chemical cross-linking data [23], which have certain limitations. Although cross-linking between two uroplakins suggests a topological relationship, the lack of cross-linking between two proteins can simply reflect a lack of suitably positioned cross-linkable amino acid residues. Independent data are therefore required to verify the selective interactions among the uroplakin proteins. In addition, previous immunogold localization of uroplakins was performed on urothelial tissues that had been fixed only mildly in order to preserve the immunoreactivity of the antigens. The ultrastructure was therefore insufficiently preserved to discern whether a particular uroplakin is present in all the urothelial plaques [17,18]. This issue is crucially important as it impacts on the validity of the existing structural models of the urothelial plaque.

In this paper, we show that treatment of intact urothelial plaques with chymotrypsin resulted in the selective digestion of the cytoplasmic tail of UPIII. We also demonstrate that when uroplakins solubilized in 2% octylglucoside were fractionated using ion-exchange resins, uroplakins could be separated into two subsets. UPII and UPIII have rather basic and acidic isoelectric points, respectively; and they were accordingly retained by the cation and anion exchangers. Interestingly, UPIa and UPIb, despite their similar isoelectric points, co-purified with UPII and UPIII, respectively, thus providing strong, independent support for the concept of uroplakin pairs. Finally, we show that UPIII was present in practically all the plaques, associated with both the urothelial apical surface and cytoplasmic fusiform vesicles, suggesting that urothelial plaques have a relatively uniform uroplakin composition.

MATERIALS AND METHODS

Antibodies

Antibodies used include rabbit antisera prepared against synthetic peptides corresponding to different domains of uroplakins as described [21,24], AE1 and AE3 antibodies against keratins [25,26] and a new mouse monoclonal antibody, AU1, to UPIII. To generate mouse monoclonal antibodies to uroplakins, we immunized mice with total bovine uroplakins, screened for hybridoma clones secreting antibodies to uroplakins by ELISA and by immunohistochemical staining of microwaved paraffin sections of formalin-fixed human ureter mucosa. AU1, an IgG1, stained specifically urothelial umbrella cells and urothelial-derived tumours [27], with no detectable staining of any non-urothelial tissues tested.

Protease digestion

Total membranes and Sarkosyl-insoluble urothelial plaques were prepared from bovine bladders according to a recently improved procedure ([28]; also see [18,24]). Purified AUMs were incubated at 25 °C overnight with chymotrypsin (Boehringer Mannheim/Roche, Mannheim, Germany) with a protease/protein ratio (w/w) of 1:25. PMSF at 1 mM and aprotinin at 10 μM (Sigma, St. Louis, MO) were used to stop the protease action. The samples were centrifuged at 16000 ***g*** at 4 °C for 30 min, and the recovered plaques were dissolved in 1% SDS.

Ion-exchange separation of uroplakins

Purified bovine plaques were dissolved in buffer A containing 2% octylglucoside, 1 mM EDTA, 1 mM EGTA and 10 mM Hepes (pH 7.4). After centrifugation at 100000 ***g*** for 1 h, the solubilized uroplakins were incubated with a cation exchanger (SP-Sepharose; Pharmacia, Piscataway, NJ, U.S.A.), which had been equilibrated with the same buffer, at room temperature for 30 min. The resins were washed with 3 vol. of buffer A, and eluted using the same buffer containing 1 M NaCl. The unbound fraction was incubated with an anion exchanger (Q-Sepharose; Pharmacia) at room temperature for 30 min, washed and eluted as before.

SDS/PAGE and immunoblot analysis

Proteins were separated electrophoretically using a 17% polyacrylamide gel (acrylamide/bisacrylamide ratio, 120:1) [29]. The separated proteins were transferred electrophoretically to nitrocellulose sheet [30], and reacted with various primary antibodies.

This was followed by incubation with peroxidase-conjugated goat anti-mouse IgG or goat anti-rabbit IgG antibodies [25,31], and visualized using an enhanced chemiluminescence (ECL) system (Pierce, Rockford, IL, U.S.A.) [28].

Immunohistochemical staining

Bovine and mouse bladder tissue blocks were fixed with formaldehyde, and embedded in paraffin [32]. The deparaffinized sections were microwaved, treated with 1% hydrogen peroxide in methanol to block the endogenous peroxidase activity, incubated with normal goat serum (1:50) in PBS, followed by incubation at 4 °C overnight with mouse monoclonal antibodies against keratins (the AE1 and AE3 antibodies [25,31]) or UPIII (the AU1 antibody). The sections were then incubated with horseradish peroxidase-conjugated goat anti-mouse IgG, incubated with the diaminobenzidine substrate, and counter-stained with haematoxylin [32].

Immunoelectron microscopy

Bovine, rat and mouse bladders were fixed for 4 h at 4 °C in a freshly prepared solution containing 3% paraformaldehyde, 0.1% glutaraldehyde, 4% sucrose and 0.1 M sodium cacodylate buffer (pH 7.4). The 10 or 15 nm gold-particle-conjugated goat anti-mouse IgG antisera (Amersham Life Science) were used for antigen detection [33].

RESULTS

Chymotryptic digestion of bovine urothelial plaques

To test the validity of the current model of urothelial plaque structure (Figure 1), we studied the protease susceptibility of the uroplakins in intact versus SDS-denatured bovine urothelial plaques. Although all four uroplakins harboured numerous chymotrypsin-sensitive sites and this enzyme could completely digest SDS-denatured uroplakins (results not shown), most uroplakins in intact urothelial plaques were highly resistant to chymotrypsin. Thus immunoblotting revealed that UPIa, UPIb and UPII remained intact even when the plaques were treated with a high concentration of chymotrypsin at a protease/substrate ratio of 1:25 (w/w; Figures 2A–2D and Table 1). The only exception was the 47 kDa UPIII, which could be converted, almost quantitatively, into a smaller fragment of ≈ 42 kDa even when the protease/substrate ratio was as low as 1:100 (Figures 2A, 2E and 2F, and results not shown). This band retained epitopes 1 and 2 that were presumed to be extracellular (Figures 2E and 2F), but lost epitopes 3 and 4 that were presumed to be intracellular (Figures 2G and 2H, and Table 1). These results indicate that all extracellular domains of uroplakins are highly protease-resistant, most likely reflecting tight protein–protein interactions within a 16 nm particle, and that chymotrypsin selectively cleaved the cytoplasmic tail of UPIII (Figure 1).

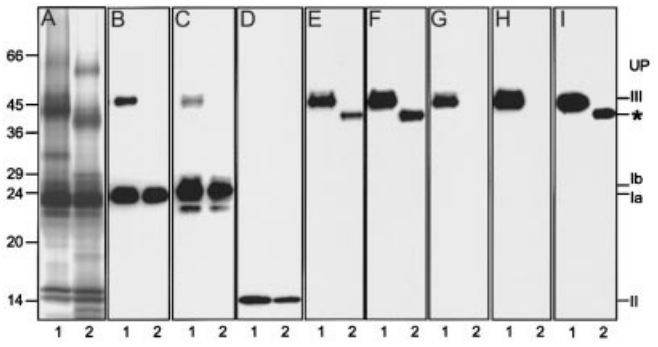

Figure 2 Selective digestion of the cytoplasmic domain of UPIII by chymotrypsin

Purified bovine urothelial plaques were treated with chymotrypsin, and the proteins recovered by centrifugation were analysed by SDS/PAGE (see Materials and methods section). Lanes 1 and 2 show the staining patterns of intact and chymotrypsin-digested urothelial plaques, respectively. The polypeptides were visualized either by silver nitrate staining (**A**), or by immunoblotting using various uroplakin antibodies (**B**–**I**) including those against: (**B**) UPIa epitope 3 (P3); (**C**) UPIb P1; (**D**) UPII P2; (**E**) UPIII P1; (**F**) UPIII P2; (**G**) UPIII P3; (**H**) UPIII P4; (**I**) an extracellular epitope of UPIII (defined by a new mouse monoclonal antibody AU1). With the exception of UPIII epitopes 3 and 4, all epitopes were presumed to be extracellular (see Figure 1). Asterisk denotes a 42 kDa degradation product of UPIII. The ≈ 50 kDa bands in (**B**) and (**C**) represent dimers of UPIa and UPIb [19,23]; these dimers may not be a part of the 16 nm particle and are thus sensitive to proteolysis. Numbers on the left denote molecular masses (kDa) of protein markers.

Table 1 Epitopes of uroplakins as defined by synthetic peptides

A total of 14 synthetic peptides corresponding to different domains of bovine UPIa, UPIb, UPII and UPIII were used to generate rabbit antisera [21,24]. The numbers on the sequences denote the amino acid positions in the protein sequences, as described earlier [19,21,22]. Abbreviations used: IB, immunoblot (+ means that the antibody reacts strongly); IH, immunohistochemical staining of paraffin sections that have been microwave-treated; CS, chymotrypsin sensitivity (see Figure 2 and text).

Peptide	Sequence	Antisera	IB	IH	CS
UPIa					
P1	^{1}MASAAAATTEKGS^{13}	143	+	−	−
P2	^{41}VTADQYRIYPLMGVSGKDD^{59}	146	+	−	−
P3	^{139}DSNQGRELTRLWDR^{152}	148	+	+	−
P4	^{176}RATTPEVVFP^{185}	150	+	−	−
UPIb					
P1	^{2}AKDDSTVRCFQGLLIFGN^{19}	260	+	−	−
P2	^{130}QNNSPPNNDDQWKNNGVTKT^{149}	262	+	−	−
UPII					
P1	^{1}ELVSVVDSGSG^{11}	155, 156	+	−	−
P2	^{7}DSGSGFTVTRLSA^{19}	044	+	−	−
P3	^{18}SAYQVTNLAPGTKYYI^{33}	157, 158	+	−	−
P4	^{40}GASTESSREIPMSTFPRRK^{58}	160	+	+	−
UPIII					
P1	^{87}STFQQTQGGRTGPYK^{101}	164	+	−	−
P2	^{179}QTLWSDPIRTDRL^{191}	182	+	+	−
P3	^{243}ATSHDSQITQEAVPK^{257}	173, 174	+	−	+
P4	^{261}TSEPSYTSVNRGPSLD^{276}	165, 166	+	−	+

Separation of UPIa/II and UPIb/III pairs by ion-exchange resins

Although UPIa and UPIb have similar isoelectric points (calculated pI, ≈ 5.3–5.8), UPII is very basic (calculated pI of the mature polypeptide, 10.84) whereas UPIII is slightly acidic (calculated pI, ≈ 4.9) [19,21,22]. The actual charge difference between UPII and UPIII is even greater because mature UPII is not glycosylated [22], while UPIII harbours about 20 kDa equivalents of negatively charged complex sugars [21]. Such a large charge difference between UPII and UPIII has been confirmed by two-dimensional gel electrophoresis [17]. One would therefore predict that UPII and UPIII should be absorbed selectively by cation- and anion-exchange resins, respectively. If this were true, it would be interesting to determine whether UPIa and UPIb could form complexes and co-purify with UPII and UPIII, respectively, as one might predict on the basis of the proposed pair relationship [23].

We have therefore solubilized bovine urothelial plaques with octylglucoside and fractionated the uroplakins using ion-

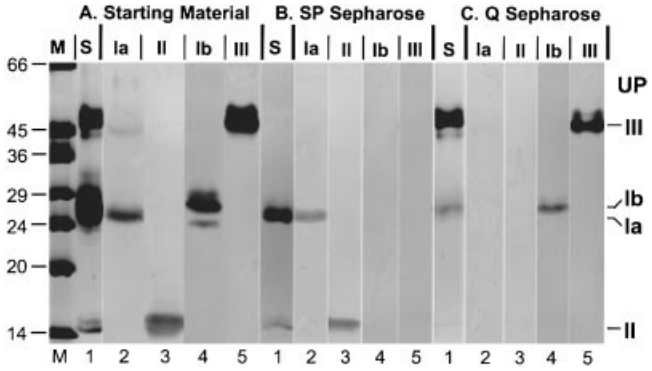

Figure 3 Fractionation of the octylglucoside-solubilized uroplakins using ion-exchange resins

(**A**–**C**) SDS/PAGE patterns of (**A**) the total uroplakins, uroplakins bound by (**B**) the cation exchanger SP-Sepharose and those bound by (**C**) the anion exchanger Q-Sepharose. The uroplakins were visualized by (lane 1) silver nitrate staining or by (lanes 2–5) immunoblotting using antibodies to (lane 2) UPIa, (lane 3) UPII, (lane 4) UPIb and (lane 5) UPIII. Note the separation of uroplakins into two sets of polypeptides, one contains UPIa/II (**B**) and the other UPIb/III (**C**). M denotes molecular-mass markers.

exchange resins (Figure 3). As reported earlier, urothelial plaques are relatively insoluble in many detergents, including Nonidet P-40, Triton X-100 and sodium Sarkosyl, but they can be readily solubilized in 2% octylglucoside [18,24,28]. The octylglucoside-solubilized uroplakins were incubated with the SP-Sepharose cation-exchange resins. After extensive washing, the bound proteins were eluted with the same buffer containing 1 M NaCl. The analysis of such proteins by SDS/PAGE revealed not only the expected presence of UPII, but also its presumed partner, UPIa; no UPIb and UPIII were detected in this fraction (Figure 3B). We then incubated the unbound fraction from the above experiment with the Q-Sepharose anion-exchange resins. The bound fraction contained, in addition to the expected acidic UPIII, its presumed partner UPIb; no UPIa and UPII were detected (Figure 3C). These results support strongly the existence of two uroplakin pairs consisting of UPIa/II and UPIb/III.

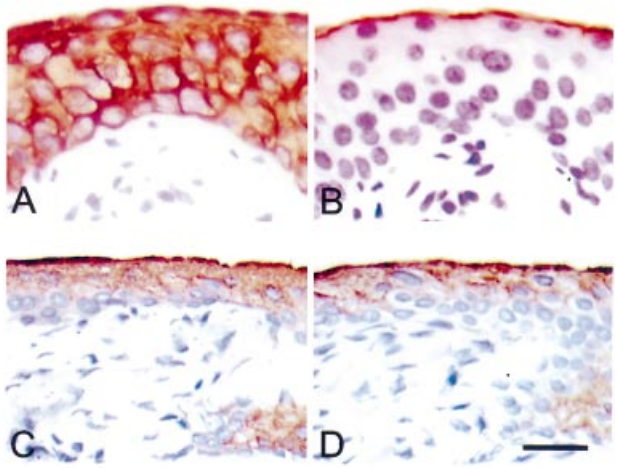

Figure 4 Immunohistochemical staining of UPIII using the AU1 monoclonal antibody

Paraffin sections of bovine (**A** and **B**) and mouse (**C** and **D**) bladders were stained by the indirect immunoperoxidase technique using (**A** and **C**) a mixture of anti-keratin antibodies AE1 and AE3, or (**B** and **D**) AU1, a new mouse monoclonal antibody to UPIII. Note the nearly uniform staining of the urothelium by the anti-keratin antibody, and the selective staining of the superficial umbrella cells by AU1. Scale bar, 50 μm.

Immunogold localization of UPIII using a new monoclonal antibody, AU1

As mentioned earlier, information on the distribution of uroplakins among various urothelial plaques is important since it allows us to assess the possible subunit heterogeneity among the plaques. The localization of uroplakins by the immunogold technique has been hampered, however, by a lack of suitable antibodies. We have previously raised rabbit antibodies against synthetic peptides corresponding to 14 epitopes of the four uroplakins [21,24]. Although all these antibodies recognized SDS-denatured uroplakins by immunoblotting (Figures 2 and 3), most of these antibodies reacted poorly with uroplakins on fixed tissue sections, probably due to an extremely compact particle structure (Table 1). Polyclonal antibodies raised against intact uroplakins were more useful for immunolocalization studies, but even these antibodies required mildly fixed tissues whose ultrastructure was poorly preserved.

We therefore generated a new mouse IgG1 monoclonal antibody, AU1, which was screened specially for its ability to stain paraffin sections of formalin-fixed tissues (Figure 4). This antibody recognized a single band of 47 kDa UPIII, even when it was used to immunoblot total cellular proteins of bovine or mouse urothelia (results not shown). Its reaction with the 42 kDa chymotryptic degradation product of UPIII indicated that its epitope resided in the extracellular domain (Figure 2I, lane 2). Immunogold labelling of ultra-thin sections of bovine (Figure 5A), rat (Figure 5B) and mouse (Figure 5D) urothelia revealed that UPIII was associated with $>90\%$ of urothelial plaques covering the entire apical surface, as well as the lumen of cytoplasmic fusiform vesicles.

DISCUSSION

In this work, we studied the organization of uroplakin subunits in urothelial plaques, which represent the major differentiation products of mammalian urothelium. We have obtained new information with regard to the accessibility of uroplakin domains towards proteases, the protein–protein interactions among uroplakin subunits, and the subunit composition of urothelial plaques.

Transmembrane topology of uroplakins

We have previously proposed a model (Figure 1) in which the single N-terminal hydrophilic domains of UPII and UPIII, as well as the two major hydrophilic domains that interconnect the first and second TMDs (TMD 1/2) and TMD 3/4 of UPIa and UPIb, are extracellular [19,20]. According to this model, UPIII is unique in that it is the only uroplakin that possesses a significant cytoplasmic domain, which we hypothesized to play a role in anchoring the urothelial plaque on to an underlying cytoskeleton [21]. Our finding that most uroplakin epitopes, located in the extracellular domains, are not accessible to the antibodies in immunolocalization studies (Table 1) suggests that these domains are either buried or interact tightly, forming the 16 nm particle. Our current data showing that these domains in intact urothelial plaques are highly resistant to chymotrypsin (Figure 2) provide strong support to this idea. Moreover, the fact that the lone cytoplasmic domain of UPIII is particularly sensitive to proteolysis (Figure 2) suggests that this domain can adopt a

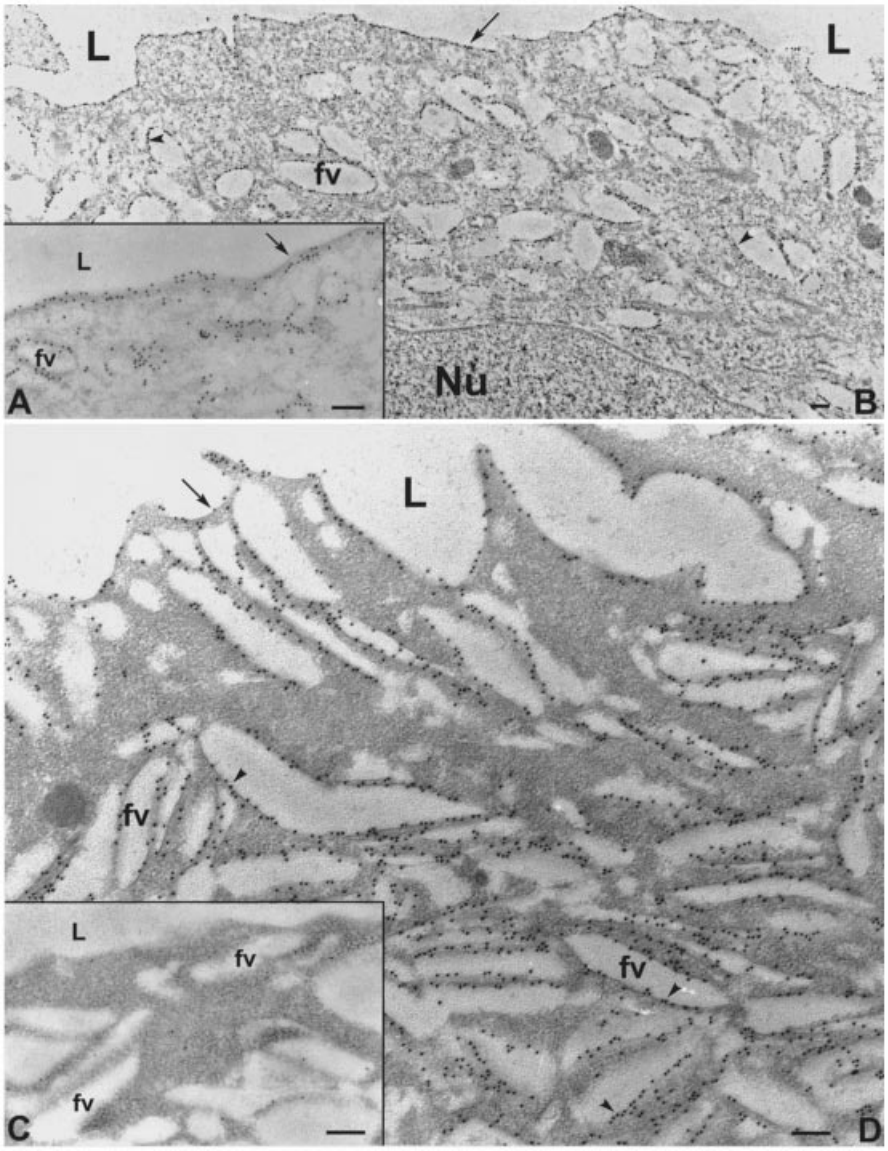

Figure 5 Electron-microscopic localization of UPIII in urothelium

(**A**) Bovine, (**B**) rat and (**C**, **D**) mouse urothelial umbrella cells were labelled with (**A**, **B** and **D**) AU1 antibody against UPIII, or (**C**) PBS buffer (negative control). Note the intense AU1-labelling of the entire urothelial apical surface (arrows) as well as practically all cytoplasmic fusiform vesicles (fv; arrowheads). Gold particles are 10 nm (**A**–**C**) or 15 nm (**D**) in diameter. L, luminal space; Nu, nucleus. Scale bars, 200 nm.

relatively open configuration, which may be important for membrane–cytoskeletal interaction.

The concept of uroplakin pairs

On the level of protein–protein interaction, our finding that UPIa and UPIb co-purified with UPII and UPIII, respectively, provides direct evidence that the four uroplakins interact selectively forming two pairs, i.e. UPIa/II and UPIb/III (Figure 3). As mentioned, although it is not surprising that the basic UPII and the relatively acidic UPIII were retained by the cation and anion exchangers, respectively, it is remarkable that UPIa and UPIb, which have about the same isoelectric point, were retained selectively by the cation and anion exchangers (Figure 3). This suggests that uroplakins were fractionated into two complexes containing UPIa/II and UPIb/III. This result is entirely consistent with our previous chemical cross-linking data showing that UPIa and Ib are cross-linked with UPII and UPIII, respectively [23]. In addition, we found recently that the ablation of the *UPIII* gene resulted in the mis-targeting of its presumed partner, UPIb, again supporting the selective interactions between these two uroplakins [34]. Taken together, these results strongly support the idea that uroplakins interact specifically, forming pairs consisting of UPIa/II and UPIb/III.

Urothelial plaque structure

Based on chemical cross-linking experiments, we have previously made several observations: (i) UPIa and UPIb tended to aggregate and form SDS-resistant homo-oligomers, suggesting that

they are particularly suitable for occupying the inner six domains of the 16 nm particle; (ii) both UPII and UPIII could be cross-linked to form homodimers but not higher oligomers, consistent with their being responsible for forming the outer six subdomains; (iii) no heterodimer of UPIa/Ib was found; (iv) UPIa and UPIb were found to cross-link, highly efficiently, to UPII and UPIII, respectively; and (v) no UPII–UPIII cross-links were found [23]. These data are consistent with at least two preliminary models of AUM structure. One of the models depicts the presence of all four uroplakins, at a fixed stoichiometric ratio, in all the 16 nm particles; this implies that all the urothelial plaques will have the same uroplakin composition. Another possibility is that the two uroplakin pairs form distinct 16 nm particles that then form two different types of plaque. This model implies that a given uroplakin may be found in only a subpopulation of plaque [23]. This two-plaque possibility now seems unlikely, because it is inconsistent with our present finding that UPIII is associated with practically all the plaques (Figure 5). This conclusion is supported by our recent finding that urothelial plaques are not static structures, but are probably highly dynamic, capable of breaking and fusing, which would result in the intermingling of the 16 nm particles from neighbouring plaques [35]. More data are needed to further define the precise location of the individual uroplakins within a 16 nm particle, and how such particles interact to form the urothelial plaques.

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