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Selective Interactions of UPIa and UPIb, Two Members of the Transmembrane 4 Superfamily, with Distinct Single Transmembrane-domained Proteins in Differentiated Urothelial Cells*

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Xue-Ru Wu‡§¶‖, Juan J. Medina‡, and Tung-Tien Sun**

*From the Departments of ‡Urology and §Microbiology, **Epithelial Biology Unit, the Ronald Perelman Department of Dermatology, and Department of Pharmacology, Kaplan Cancer Center, New York University School of Medicine, and the ¶Veterans Administration Medical Center in Manhattan, New York, New York 10016*

The transmembrane 4 (TM4) superfamily contains many important leukocyte differentiation-related surface proteins including CD9, CD37, CD53, and CD81; tumor-associated antigens including CD63/ME491, CO-029, and SAS; and a newly identified metastasis suppressor gene *R2*. Relatively little is known, however, about the structure and aggregation state of these four transmembrane-domained proteins. The asymmetrical unit membrane (AUM), believed to play a major role in stabilizing the apical surface of mammalian urothelium thus preventing it from rupturing during bladder distention, contains two TM4 members, the uroplakins (UPs) Ia and Ib. In association with two other (single transmembrane-domained) membrane proteins, UPII and UPIII, UPIa and UPIb form 16-nm particles that naturally form two-dimensional crystalline arrays, thus providing unique opportunities for studying membrane structure and function. To better understand how these proteins interact to form the 16-nm particles, we analyzed their nearest neighbor relationship by chemical cross-linking. We show here that UPIa and UPIb, which share 39% of their amino acid sequence, are cross-linked to UPII and UPIII, respectively. We also show that UPIa has a propensity to oligomerize, forming complexes that are stable in SDS, and that UPII can be readily cross-linked to form homodimers. The formation of UPII homodimers is sensitive, however, to octyl glucoside that can solubilize the AUMs. These data suggest that there exist two types of 16-nm AUM particles that contain UPIa/UPII or UPIb/UPIII, and support a model in which the UPIa and UPII occupy the inner and outer domains, respectively, of the UPIa/UPII particle. This model can account for the apparent "redundancy" of the uroplakins, as the structurally related UPIa and UPIb, by interacting with different partners, may play different roles in AUM formation. The model also suggests that AUM plaques with different uroplakin compositions may differ in their assembly, and in their abilities to interact with an underlying cytoskeleton. Our data indicate that two closely related TM4 proteins, UPIa and UPIb, can be present in the same cell, interacting with distinct partners. AUM thus provides an excellent model system for studying the targeting, processing, and assembly of TM4 proteins.

The "transmembrane four" (TM4)[1] superfamily is a recently described gene family that encodes a group of cell surface proteins all possessing four conserved transmembrane domains. Members of this family are found in lymphocytic, mesenchymal, and epithelial cells (reviewed by Horejsi and Vlcek (1991) and Wright and Tomlinson (1994)). Thus the TM4 proteins that have been identified so far include several tumor-related surface proteins CO-029 (Szala *et al.*, 1990), L6 (Marken *et al.*, 1992), SAS (Jankowski *et al.*, 1994), and R2 (Gaugitsch *et al.*, 1991) (the last was recently described as a metastasis suppressor gene for prostate cancer (Dong *et al.*, 1995)); leukocyte-differentiation markers CD9 (Boucheix *et al.*, 1991; Lanza *et al.*, 1991), CD37 (Classon *et al.*, 1990), CD53 (Amiot, 1990; Korinek and Horejsi, 1993), CD63 (Hotta *et al.*, 1988; Metzelaar *et al.*, 1991), CD81 (also known as TAPA-1; Oren *et al.*, 1990; Engel and Tedder, 1994), and CD82 (Lebel *et al.*, 1994; Nagira *et al.*, 1994); as well as major epithelial differentiation products uroplakins Ia and Ib (Yu *et al.*, 1994). Closely related molecules, SM23 and SJ23, have even been found in parasitic helminth schistosomes (Davern *et al.*, 1991; Reynolds *et al.*, 1992), indicating that members of this gene family are conserved during evolution. Since the intron positions of several of the TM4 genes are conserved, these genes may have diverged from a common ancestral gene (Horejsi and Vlcek, 1991; Wright *et al.*, 1993; Wright and Tomlinson, 1994). Recent data indicate that some of these TM4 proteins may play important roles in cell growth, adhesion, and metastasis (Horejsi and Vlcek, 1991; Miyake *et al.*, 1991; Schick and Levy, 1993; Wright and Tomlinson, 1994; Dong *et al.*, 1995). However, many crucial questions regarding the structure and function of TM4 proteins remain unanswered. For example, how do these integral membrane proteins, most of them lacking a significant cytoplasmic domain, perform their functions on the cell surface? In several cases, more than one TM4 protein exists in the same cell; thus CD81 and CD82 coexist in T cells (Imai and Yoshie, 1993; Nagira *et al.*, 1994), and uroplakins Ia and Ib coexist in the differentiated urothelial cells (Yu *et al.*, 1994). In such cases, do these TM4 proteins always interact with each other? Or do some of these TM4 proteins interact specifically

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‖ To whom correspondence should be addressed: Dept. of Urology, New York University Medical School, 550 First Ave., New York, NY 10016. Tel.: 212-263-5316; Fax: 212-263-8561.

[1] The abbreviations used are: TM4, transmembrane 4; EGS, ethylene glyco-*bis*(succinimidyl succinate); DFDNB, 1,5-difluoro-2,4-dinitrobenzene; PAGE, polyacrylamide gel electrophoresis; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propaneslfonic acid; DTR, diphtheria toxin receptor; HB-EGF, heparin-binding epidermal growth factor.

with other integral membrane proteins?

Uroplakins Ia (UPIa; 27-kDa[2]) and Ib (UPIb; 28-kDa; also known as TI-1) (Kallin *et al.*, 1991) are two newly identified TM4 proteins that are synthesized by the terminally differentiated, superficially located cells of mammalian urothelium (Yu *et al.*, 1994). Together with two other proteins, *i.e.* the 15-kDa uroplakin II (Lin *et al.*, 1994, 1995) and the 47-kDa uroplakin III (Wu and Sun, 1993), the UPIa and UPIb are major protein components of the so-called asymmetrical unit membrane (AUM) (Wu *et al.*, 1990; Yu *et al.*, 1990), which forms numerous plaques covering about 80% of the apical surface area of mammalian urothelium (Porter and Bonneville, 1963; Hicks, 1965; Porter *et al.*, 1967; Koss, 1969; Chlapowski *et al.*, 1972). These AUM plaques are believed to play a role in stabilizing the luminal surface of the epithelium thus preventing it from rupturing during bladder distention (Staehelin *et al.*, 1972; Minsky and Chlapowski, 1978; Sarikas and Chlapowski, 1986). Recent biochemical data indicate that the major hydrophilic loop interconnecting the third and fourth trans-membrane domains of UPIa and UPIb is exposed on the extracellular surface, because this domain becomes protected from protease digestion once the *in vitro* transcribed and translated UPIa is inserted into dog pancreatic microsomes (Yu *et al.*, 1994). In addition, in both UPIa and UPIb, this domain contains an *N*-glycosylation site which harbors high mannose type carbohydrates (Wu *et al.*, 1994; Yu *et al.*, 1994). These data strongly suggest that UPIa and UPIb, like several other members of the TM4 superfamily, assume the so-called "type III" transmembrane configuration with the major hydrophilic domain extending into the extracellular space leaving very little cytoplasmic domains (Yu *et al.*, 1994).

The asymmetrical unit membrane offers unique opportunities for studying the detailed structural arrangement and possible function of the two differentiation-dependent members of the TM4 family, *i.e.* the uroplakins Ia and Ib, because AUMs can be purified in milligram quantities (Wu *et al.*, 1990; 1994). Moreover, uroplakins interact closely with one another forming highly organized 16-nm protein particles that naturally form two-dimensional crystalline arrays thus greatly facilitating a detailed analysis of protein structure (Hicks and Ketterer, 1969; Vergara *et al.*, 1969; Chlapowski *et al.*, 1972; Taylor and Robertson, 1984; Walz *et al.*, 1995). We have therefore probed the topographical relationship among the four major integral membrane protein subunits of the asymmetrical unit membrane using the chemical cross-linking approach. Unexpectedly, our results indicate that uroplakins Ia and Ib are cross-linked to the 15-kDa uroplakin II and the 47-kDa UPIII, respectively. The fact that the two structurally related uroplakins Ia and Ib are cross-linked to different partners suggests that the two TM4 proteins play distinct roles in AUM structure. In addition, we present data showing that, in intact AUMs, uroplakin II can be cross-linked to form a homodimer, and that UPIa can form oligomers that are stable in SDS. Taken together, these results suggest a model in which uroplakins Ia and II occupy the inner and outer domains, respectively, of a 16-nm protein particle, and raise the possibility that AUMs are composed of two types of 16-nm particles containing different subsets of uroplakin molecules.

[2] These apparent molecular masses of uroplakins were determined by analyzing nonreduced uroplakins by SDS-PAGE on a 17% polyacrylamide gel (Wu *et al.*, 1990; Yu *et al.*, 1990). These values varied slightly with sample reduction and with the use of different electrophoretic conditions, such as Tricine-SDS-PAGE. These relative sizes, along with the cDNA-derived molecular masses, of uroplakin monomers as well as cross-linked dimers are summarized in Table I.

MATERIALS AND METHODS

Isolation of Asymmetric Unit Membranes—To isolate the crude membranes of bovine urothelium, we obtained bovine bladder mucosa by scraping, washed the cells three times in phosphate-buffered saline, and homogenized them in 10 mM Hepes/NaOH (pH 7.5) containing 1 mM each of phenylmethylsulfonyl fluoride, EDTA, and EGTA. After centrifugation at 2,000 × *g* at 4 °C for 10 min, the pellet was homogenized in the same buffer, transferred onto a 1.6 M sucrose cushion, and centrifuged at 28,000 × *g* at 4 °C for 20 min. The membranes located at the interface were collected, washed with 10 mM Hepes (pH 7.5), and were used as the so-called "crude membrane fraction" for some of the cross-linking experiments. To further purify the asymmetrical unit membranes, we suspended these crude membranes in 2% Sarkosyl in 10 mM Hepes buffer (pH 7.5) at room temperature for 10 min and recovered the (insoluble) asymmetrical unit membranes by centrifugation at 18,000 × *g* at 4 °C for 30 min (Wu *et al.*, 1990, 1994). A portion of the AUMs was further treated with 25 mM NaOH, followed by washing with 10 mM Hepes buffer (pH 7.5). The membrane proteins were dissolved in 1% SDS and quantitated using the BCA reagent (Pierce).

Generation of Monospecific Antibodies to Uroplakins—Rabbit antisera were raised against oligopeptides that were synthesized according to the cDNA-deduced amino acid sequence of bovine uroplakins Ia (DSNQGRELTRLWDR<u>C</u>); uroplakin Ib (AKDDSSVRSFQGLLIFGN<u>C</u>); uroplakin II (<u>C</u>DSGSGFTVTRLSA and SAYQVTNLAPGTKYYI<u>C</u>); and uroplakin III (<u>C</u>ATSHDSQITQEAVPK). The underlined, terminal cysteine residues were added so that these peptides could be conjugated to keyhole limpet hemocyanin or bovine serum albumin using *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester as a cross-linking reagent. The carrier-peptide conjugates were used to immunize rabbits as described earlier (Wu and Sun, 1993).

Cross-linking of Uroplakins—Bifunctional cross-linking reagents, including EGS, sulfo-EGS, and DFDNB, were purchased from Pierce. They were dissolved immediately before use in dry Me_2SO to make a 25 mM stock solution (Abdella *et al.*, 1979), aliquots of which were then added to a membrane suspension containing 0.1 mg of protein per ml of 10 mM Hepes/NaOH buffer (pH 7.5). After 2 h at room temperature, the reaction was quenched by adding 1 M Tris/HCl (pH 7.4) to a final concentration of 50 mM. The cross-linked reaction mixtures were stored at −20 °C until further analysis.

Cleavage and Two-dimensional Electrophoretic Analyses of the Cross-linked Dimers—EGS cross-linked membrane proteins were dissolved in 1% SDS, separated by SDS-PAGE on a 17% polyacrylamide gel (acrylamide/bisacrylamide ratio, 120:1), and stained with Coomassie Blue followed by destaining in 50% methanol and 7% acetic acid. The lanes containing cross-linked proteins were incubated in 1 M NH_2OH in 50 mM sodium phosphate buffer (pH 8.5) at 37 °C for 6 h. The gel piece was then equilibrated in SDS sample buffer (50 mM Tris/HCl, pH 6.8; 2% SDS; 10% glycerol, and 5% β-mercaptoethanol) at room temperature for 30 min and subjected to a second dimensional SDS-PAGE (same condition as the first dimension).

Silver Nitrate Staining and Immunoblotting—The polyacrylamide gel was fixed in a solution containing 50% methanol and 7% acetic acid for 2 h, soaked in 10% glutaraldehyde for 30 min, washed extensively in distilled water, and then exposed to 20% silver nitrate dissolved in 0.4% NaOH, 0.1% NH_4OH, and 2% ethanol for 8 min. After washing in distilled water for 1 h, the gel was incubated in a solution containing 0.02% formaldehyde, 10% ethanol, and 0.005% citric acid. For immunoblotting, proteins separated by SDS-PAGE were electrophoretically transferred to a nitrocellulose paper. After the unoccupied sites of the paper were blocked with 5% milk in phosphate-buffered saline, the paper was incubated with rabbit antibodies against individual uroplakins followed by a peroxidase-conjugated goat-anti-rabbit antibody (Surya *et al.*, 1990).

RESULTS

Cross-linking of Crude Urothelial Membranes—We showed previously that by using a combination of differential centrifugation and selective removal of contaminating non-AUM vesicles with certain detergents, we could isolate large quantities of highly purified bovine urothelial AUMs (Wu *et al.*, 1990, 1994). Although these AUMs morphologically resembled the urothelial plaques found *in situ*, we could not rule out the possibility that the isolation procedure, which involved relatively harsh treatments such as washing with Sarkosyl and a high pH solution, might have altered the AUM structure. We also could not rule out the possibility that certain AUM-asso-

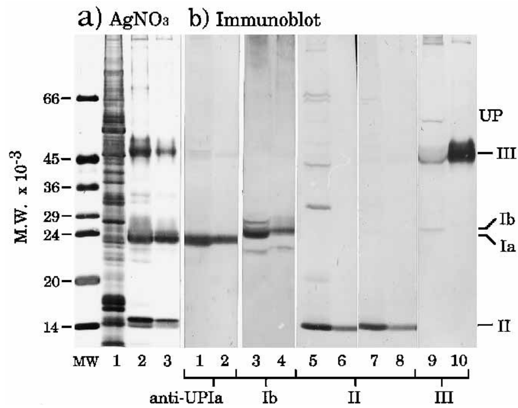

FIG. 1. **Specificity of antibodies against individual bovine uroplakins.** *a*, proteins of crude bovine urothelial membranes (*lane 1*), Sarkosyl-washed AUMs (*lane 2*), and additionally NaOH-washed AUMs (*lane 3*) were dissolved in 1% SDS at room temperature, resolved by SDS-PAGE, and visualized by silver nitrate (*$AgNO_3$*) staining. *b*, proteins of crude urothelial membranes (*odd-numbered lanes*) and Sarkosyl-washed AUMs (*even-numbered lanes*) were electrophoretically transferred to nitrocellulose and immunoblotted using (*lanes 1* and *2*) antibodies against a synthetic oligopeptide of UPIa; (*3* and *4*) anti-UPIb; (*5* and *6*) anti-UPII; (*7* and *8*) another anti-UPII; and (*9* and *10*) anti-UPIII. For the sequences of the synthetic oligopeptides, see "Materials and Methods." Numbers on the *left* denote the molecular weights (*M.W.*) of standard proteins. The relative positions of the four major uroplakins (the 27-kDa UPIa, the 28-kDa UPIb, the 15-kDa UPII, and the 47-kDa UPIII) are marked on the *right*. Note that most of the antibodies are monospecific for their respective uroplakin antigens (see text).

ciated proteins might have been stripped off. To alleviate these problems, we decided to begin by analyzing the neighboring relationship of the uroplakins in native urothelial membranes that had not experienced the detergent/alkaline treatments. We could accomplish this by isolating crude membranes from bovine urothelium, cross-linking their proteins using EGS, a bifunctional reagent that cross-links neighboring amino groups, and monitoring the cross-linked status of individual uroplakins by immunoblotting.

A prerequisite of this approach was the availability of antibodies that were monospecific for individual uroplakin molecules. We therefore raised a panel of rabbit antisera against synthetic peptides corresponding to sequences of the four major uroplakins. Immunoblotting established that all these antisera reacted strongly with their respective uroplakins (Fig. 1). Antisera to uroplakins Ia, II, and III recognized well defined 27-, 15-, and 47-kDa protein bands, respectively (Fig. 1*b*). Antisera to uroplakin Ib recognized multiple bands in the molecular mass range of 25 to 28 kDa; however, this apparent heterogeneity could be completely accounted for by glycosylation (Wu *et al.*, 1994; Yu *et al.*, 1994). Moreover, at least one antiserum for each uroplakin was shown to recognize, specifically, only the corresponding uroplakin in the crude urothelial membrane fraction (Fig. 1).

These monospecific antisera enabled us to monitor the cross-linking status of the uroplakins that were present in crude urothelial membranes that had been treated with various concentrations of EGS (Fig. 2). This experiment revealed the formation of three well defined, cross-linked uroplakin species. A new 22-kDa band was recognized only by the uroplakin II antibody and was therefore presumably a UPII homodimer; a 35-kDa band was recognized by antisera to both UPII and UPIa and was thus likely a heterodimer of UPIa and UPII; and finally a 72-kDa band reacted with antisera to both UPIb and UPIII, suggesting a UPIb/UPIII heterodimer (Fig. 2). Similar results were obtained using a hydrophilic analogue of EGS, the sulfo-EGS, although the yield of the UPIa/UPII heterodimer was greatly reduced (Fig. 3).

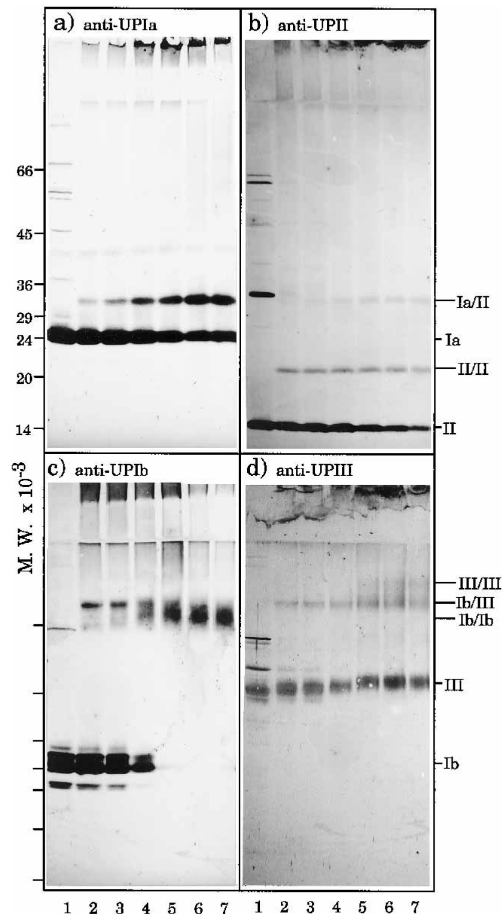

FIG. 2. **Chemical cross-linking of uroplakins that are present in native urothelial membranes.** Crude bovine urothelial membranes were cross-linked with (*lane 1*) 0, (*2*) 0.5, (*3*) 1, (*4*) 2, (*5*) 3, (*6*) 4, and (*7*) 5 mM EGS. Their proteins were dissolved in 1% SDS, resolved by SDS-PAGE, electrophoretically transferred to nitrocellulose, and then immunoblotted with (*a*) antibodies against UPIa (anti-UPIa), (*b*) anti-UPII, (*c*) anti-UPIb, and (*d*) anti-UPIII. Note in *Panels a*) and *b* the formation of a cross-linked 35-kDa band that was recognized by both anti-UPIa and anti-UPII (labeled *Ia/II*), and a 22-kDa band recognized only by anti-UPII (labeled *II/II*). Also note in *Panels c* and *d* the formation of a 72-kDa band recognized by both anti-UPIb and anti-UPIII (labeled *Ib/III*), a 66–68-kDa band recognized by anti-UPIb, and a 74-kDa band recognized by anti-UPIII. The relative positions of molecular weight (*M.W.*) standards, as well as those of various uroplakin monomers and dimers are shown on the *left* and *right*, respectively.

Cross-linking of Purified AUMs—To see whether the same topological relationships existed in the purified AUMs, we prepared a batch of bovine AUMs that had been washed with Sarkosyl. As we showed earlier, these isolated AUMs contained predominantly the 27-kDa UPIa, the 28-kDa UPIb, the 15-kDa UPII, and the 47-kDa UPIII (Fig. 1*a*, *lane 2*; Table I) (Lin *et al.*, 1994; Wu *et al.*, 1994; Yu *et al.*, 1994). An additional wash of these AUMs with 25 mM NaOH further reduced the level of two minor contaminant bands of 68 and 34 kDa (Fig. 1*a*, *lanes 2* and *3*). Immunoblotting of these purified AUM preparations revealed a 48-kDa, UPIa-related protein (Fig. 4*a*, *lanes 1* and *2*), which as we had reported earlier represented a homodimer of UPIa which was found in various quantities in AUM preparations (Wu *et al.*, 1994) (also see below). Cross-linking of these AUM preparations using EGS resulted in the formation of a 22-kDa UPII homodimer, a 35-kDa UPIa/UPII heterodimer, and a 48-kDa UPIa/UPIa homodimer (Fig. 4), thus confirming and extending some of the crude membrane data (Figs. 2 and 3).

So far we identified the cross-linked uroplakin species based on their relative sizes and their immunoreactivities with various antibodies to uroplakins. To confirm these assignments, we resolved the cross-linked AUM proteins by SDS-PAGE, cut out the entire gel lane containing the cross-linked uroplakins, cleaved them by incubating the gel strip in 1 M NH_2OH, and resolved the released uroplakins by a second dimensional SDS-PAGE. In this procedure, only the monomers that were released from a cross-linked product during the hydroxylamine step would migrate below the diagonal (Fig. 5*a*). Such an analysis revealed the existence of a 35-kDa, EGS cross-linked species which was cleaved by a hydroxylamine releasing a 27-kDa uroplakin Ia plus a 15-kDa uroplakin II (Fig. 5; see the circled protein spots connected by a *dashed line*), thus confirming the identity of the UPIa/UPII heterodimer. The results also clearly established the presence of a 22-kDa cross-linked product that, upon hydroxylamine treatment, released only a 15-kDa uroplakin II, thus confirming the identity of the uroplakin II homodimer (Fig. 5; *dotted line*). Finally, we observed a series of UPIa oligomers of 48 kDa (dimer) and 70 kDa (trimer), which apparently were formed during the hydroxylamine treatment (Fig. 5, *a*, *horizontal arrows*, and *b*).

Effects of Detergents and the Chain Length of Cross-linking Reagents on Uroplakin Cross-linking—We have shown recently using image enhancement techniques that each of the six outer domains of the 16-nm protein particle of AUMs is actually "connected," via some fine bridges, with an outer domain of a neighboring particle, and we proposed that this extensive network of inter-particle connections may account for the remarkable insolubility of AUMs in a large number of detergents including Nonidet P-40, CHAPS, deoxycholate, and Sarkosyl (Wu, *et al.*, 1990; Walz *et al.*, 1995). The AUMs could be solubilized, however, to some extent by Triton X-100 and almost completely by octyl glucoside (Wu *et al.*, 1990). It would therefore be of interest to see whether these latter two detergents can disrupt, perhaps to different degrees, certain uroplakin interactions as defined by cross-linking. To test this, we treated both crude urothelial membranes and highly purified AUMs with these two detergents and cross-linked the uroplakins using EGS. Immunoblotting showed that Triton X-100 had negligible effects on uroplakin cross-linkings (Figs. 6, *a* and *b*, and 7). In contrast, octyl glucoside greatly reduced the yield of cross-linking of the uroplakin II homodimer, even though it had almost no effect on the formation of the UPIa/UPII heterodimer (Figs. 6, *a* and *b*, and 7).

To assess the relative distance of the cross-linked ε-lysine groups, we treated purified AUMs with DFDNB, which has an arm length of only 3 Å (*versus* the 16 Å of EGS and sulfo-EGS). Like EGS, DFDNB cross-linked the UPIa/UPII heterodimer and UPIa/UPIa homodimer. However, it failed to cross-link the UPII/UPII dimer (Fig. 6*c*), suggesting that the ε-lysines in UPII/UPII cross-linking were >3 Å apart.

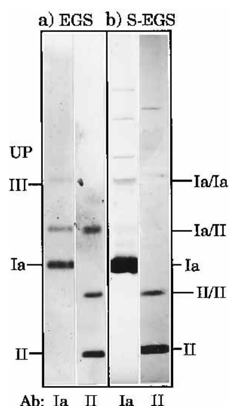

FIG. 3. **Cross-linking of crude urothelial membranes using hydrophobic *versus* hydrophilic bifunctional cross-linking reagents.** Crude bovine urothelial membranes were cross-linked with (*a*) EGS and (*b*) its hydrophilic analog, sulfo-EGS (*S-EGS*). The proteins of these cross-linked membranes were separated by SDS-PAGE and subjected to immunoblotting using anti-UPIa and anti-UPII, as indicated. Note that these two reagents are equally effective in generating the 22-kDa UPII homodimer (*II/II*); however, only the hydrophobic EGS yielded the UPIa/II heterodimer. The positions of various uroplakin monomers and dimers are marked on the *sides*.

DISCUSSION

We have probed the topographical relationship of the uroplakins in the asymmetrical unit membrane using bifunctional cross-linking reagents. The results that we have obtained so far have several important features. First, we identified the same set of cross-linked uroplakin dimers, regardless whether we used the purified AUMs (Figs. 4 and 5), or the crude urothelial membranes (Figs. 2 and 3), as our starting material. This suggests that the topographical relationships that exist in the relatively unperturbed, crude urothelial membranes must have been maintained to a large extent in our purified AUMs. Second, the cross-linking patterns were highly reproducible over a wide range of experimental conditions covering different types and concentrations of the cross-linking reagents (Figs. 2–5). Moreover, the cross-linking was highly efficient capable of capturing >30% of the uroplakin monomers using the reagent concentrations that we have tested (Figs. 2–4), thus making it less likely that we are observing the cross-linking of uroplakins entrapped in a minor AUM conformation. Third, the cross-linking of purified AUMs resulted in the formation of only a few, major protein complexes that have been identified as containing purely uroplakins. Thus the cross-linking of UPIa with EGS yielded almost exclusively the UPIa·UPII complex, and UPII yielded predominantly the UPIa/UPII heterodimer and the UPII homodimer (Figs. 2–4). Such a relatively simple cross-linking pattern of the purified AUMs is perhaps to be expected, given the fact that AUMs are known to contain only four major protein subunits (Wu *et al.*, 1990, 1994; Yu *et al.*, 1994). It was unexpected, however, that crude urothelial mem-

TABLE I
Relative sizes ($M_r \times 10^{-3}$) of uroplakins before and after chemical cross-linking

Uroplakin	SDS-PAGE		Tricine-SDS-PAGE[a]	cDNA-deduced	Sugar	Cross-linked with			
	Nonreduced	Reduced				Ia	Ib	II	III
Ia	27	24	24	28.9[b]	2–3	48			
Ib	28	27	27	29.7[b]	2–3	ND[c]	?		
II	15	15	11	10.6[d]	0	35	ND	22	
III	47	47	47	28.9[e]	18	ND[c]	72	ND	74

[a] Unpublished data.
[b] Yu *et al.* (1994).
[c] ND, not detectable.
[d] Lin *et al.* (1994).
[e] Wu *et al.* (1993).

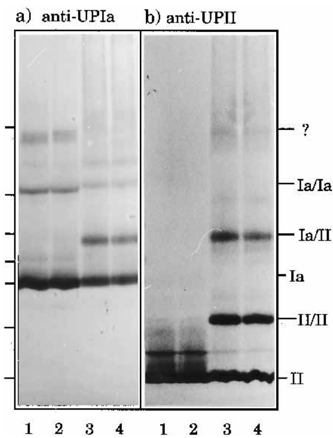

FIG. 4. **Chemical cross-linking of uroplakins that are present in purified AUMs.** Sarkosyl-washed AUMs (*lanes 1* and *3*) and the AUMs that had been further washed with NaOH (*lanes 2* and *4*) were incubated with only a buffer (*lanes 1* and *2*) or with 5 mM EGS (*lanes 3* and *4*). Proteins of these membranes were immunoblotted with (*a*) anti-UPIa and with (*b*) anti-UPII. Note the generation of the UPII/UPII homodimer, the UPIa/UPII heterodimer, and a UPIa/UPIa homodimer, thus confirming the crude membrane results. A 65-kDa species (*?*) may represent a UPIa oligomer (see Fig. 5*a* and text).

branes yielded no additional cross-linked species, although of course this negative finding does not rule out additional protein:protein interactions that may exist *in situ*. Taken together, our results strongly suggest that the uroplakin pairs that we have identified so far by the cross-linking approach reflect important protein:protein interactions that occur in the asymmetrical unit membrane.

Uroplakin Interactions: A Nearest Neighbor Analysis

Our data thus established the existence of several uroplakin pairs including homodimers and heterodimers. In addition, we found that UPIa has a tendency to oligomerize, forming complexes that are stable in SDS. The fact that UPIa and UPIb, two structurally related molecules, were cross-linked to two different uroplakin partners indicated a high degree of specificity in uroplakin interaction. The apparent molecular weights of various uroplakin monomers and cross-linked dimers are summarized in Table I.

The UPIa/UPII Heterodimer Formation—This cross-linked pair ran as a well defined, 35-kDa band during SDS-PAGE (Figs. 2–7; Table I); it reacted strongly with antisera to uroplakins Ia and II (Figs. 2–4); and it was cleaved by hydroxylamine giving rise to UPIa and UPII (Fig. 5). The apparent molecular weight of this cross-linked product was slightly smaller than the sum of its monomers (35 kDa *versus* 42 kDa), which is a common occurrence. The fact that this UPIa/UPII heterodimer was detected as a major cross-linked product of not only the purified AUMs, but also the crude urothelial membranes (Figs. 2–4), suggests that the UPIa:UPII interaction that is detected here via the cross-linking reaction is likely to be physiological. This uroplakin pair can even be generated with DFDNB, a much shorter cross-linking reagent with an arm length of only 3Å (*versus* 16 Å of EGS; Fig. 6*c*), suggesting a close proximity of the cross-linked ϵ-amino groups. On the other hand, the hydrophilic analogue of EGS, *i.e.* the sulfo-EGS, failed to cross-link this particular uroplakin pair, even though it could efficiently cross-link some other uroplakins (see below). This suggests that the reactive lysines may be embedded at least in part in a hydrophobic environment. Taken together, these results clearly established that uroplakin Ia, a member of the TM4 superfamily, interacts closely with uroplakin II, a 15-kDa "type I" integral membrane protein that is

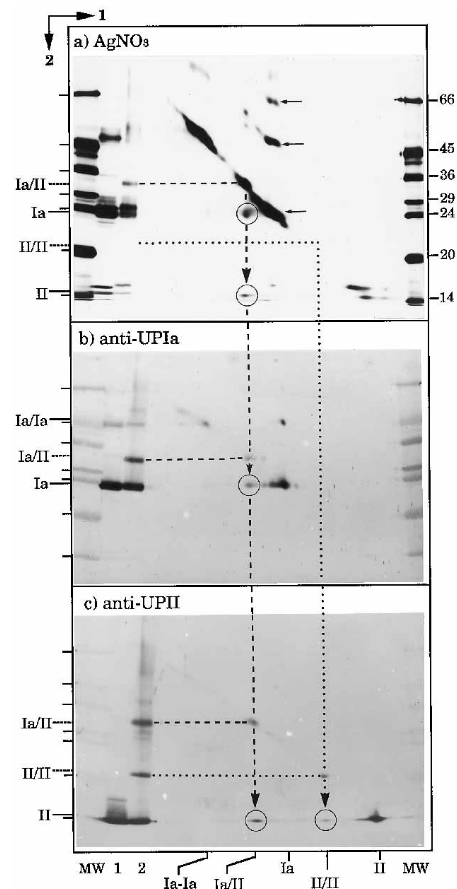

FIG. 5. **Identification of the cross-linked uroplakin complexes by two-dimensional, diagonal gel electrophoreses.** The proteins of EGS cross-linked AUMs were dissolved in 1% SDS and separated by first dimensional SDS-PAGE. After staining with Coomassie Blue and destaining, individual gel lanes were excised, incubated in 1 M hydroxylamine to cleave the cross-linked species, and subjected to a second dimensional (slab) SDS-PAGE. The two-dimensional gels were then (*a*) stained with silver nitrate (*AgNO$_3$*), or immunoblotted with (*b*) anti-UPIa or (*c*) anti-UPII. *Lanes 1* and *2* are side lanes showing the proteins of either control AUMs (*lane 1*) or EGS cross-linked AUMs (*lane 2*) that were resolved only during the second dimensional SDS-PAGE. *Arrows* marked with *1* and *2* denote the directions of the first and second dimensional SDS-PAGE. The molecular weights (*MW*) of the marker proteins are indicated on the *right* of *Panel a*. Note the cleavage of a 35-kDa cross-linked protein (*lane 2*), giving rise to a 27-kDa UPIa and a 15-kDa UPII (*dashed lines*). Also note the cleavage of a 22-kDa cross-linked protein yielding a 15-kDa UPII (*dotted lines*). A series of UPIa-related spots (*horizontal arrows*), that can be seen above the diagonal in *Panels a* and *b*, represent oligomerized UPIa (see text).

anchored into the lipid bilayer via its single transmembrane domain located at its C terminus (Lin *et al.*, 1994).

The Uroplakin II Homodimer—This cross-linked species ran as a well defined band of 22-kDa during SDS-PAGE (Figs. 2–4; Table I), it reacted with only anti-UPII (Figs. 2–5), and it was cleaved by hydroxylamine yielding exclusively a monomeric UPII (Fig. 5). Both EGS and its hydrophilic analogue, the sulfo-EGS, worked well in generating this UPII homodimer (Fig. 3), indicating that the cross-linking reaction was relatively insensitive to the hydrophobicity of the cross-linking reagent. On the other hand, this dimer formation was highly dependent on the chain length of the cross-linking reagent; while EGS and S-EGS (16 Å) worked well, the shorter DFDNB (3 Å) was ineffective (Fig. 6*c*). Interestingly, the cross-linking of UPII homodimer was abolished by octyl glucoside, which could effectively dissolve the AUMs, but this cross-linking was only marginally affected by Triton X-100 that only partially solubilized the AUMs.

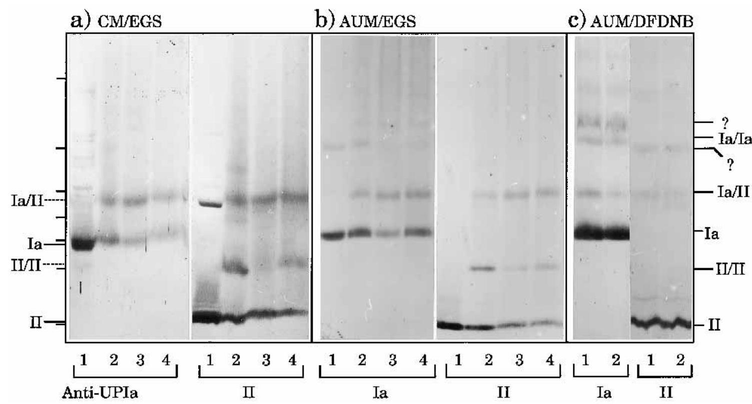

FIG. 6. **Effects of detergents and chain length of the cross-linking reagents on uroplakin cross-linking.** Cross-linking was carried out on total or crude membranes (*a*) or highly purified AUMs (*b* and *c*), using either EGS (16 Å; *Panels a* and *b*) or DFDNB (3 Å; *Panel c*). The cross-linked membrane proteins were dissolved in 1% SDS, resolved by SDS-PAGE, and immunoblotted using anti-UPIa or anti-UPII, as indicated. In *Panels a* and *b*, *lanes 1* are controls without cross-linking. EGS cross-linking was carried out in 10 mM Hepes buffer (*lanes 2*), or in the same buffer containing 2% octyl glucoside (*lanes 3*) or 2% Triton X-100 (*lanes 4*). Note that the yield of UPIa/UPII heterodimer is not affected by the detergents (*lanes 2–4*); however, the formation of UPII homodimer was largely abolished by octyl glucoside (*lanes 3*), although unaffected by Triton X-100 (*lanes 4*; see Fig. 7 for the scanning of these lanes). Also note, in *Panel c*, that the short armed DFDNB failed to produce the UPII homodimer, although it yielded the UPIa/UPII heterodimer. The relative positions of uroplakin monomer and dimer are indicated on the *sides*.

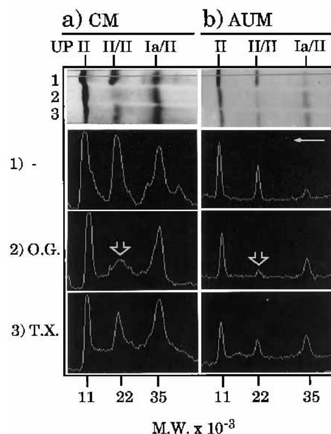

FIG. 7. **Octyl glucoside abolishes selectively the formation of uroplakin II homodimer.** *Lanes 2–4* of the immunoblots produced with anti-UPII, as shown in Fig. 6, *a* and *b*, were scanned for densitometry using a Universal Imaging Program. The samples correspond to (*a*) crude membranes and (*b*) purified AUMs that have been EGS cross-linked (*1*) without detergent (−), (*2*) with 2% octyl glucoside (*O.G.*), or (*3*) with 2% Triton X-100 (*T.X.*). The *small, white arrow* indicates the direction of SDS-gel electrophoresis, and the *large, open arrows* mark the positions of the UPII homodimer. Note that Triton had relatively little effect on uroplakin cross-linking, while octyl glucoside greatly reduced the formation of the UPII homodimer in both crude membrane and purified AUMs.

These results indicate that UPII is involved in two different kinds of protein:protein interactions. Its binding to UPIa is short-ranged as they can be cross-linked not only by EGS but also by the 3 Å DFDNB, and this binding is relatively strong as it is stable in octyl glucoside. In contrast, the binding of UPII to another UPII is relatively distant as the cross-linking required a long-armed reagent, and the binding is relatively weak as it can be disrupted by octyl glucoside. This raises the possibility that UPII interacts with UPIa within a 16-nm particle, but with UPII of perhaps another particle (see below). This also raises the possibility that a detergent's ability to break the UPII:UPII interaction, which may be involved in bridging the neighboring 16-nm particles (see below), enables the detergent to solubilize the AUMs.

The Uroplakin Ia Oligomers—Some integral membrane proteins can form complexes that are so stable that they migrate as well defined oligomers during SDS-PAGE (Lemmon *et al.*, 1992; Treutlein *et al.*, 1992; Arkin *et al.*, 1994). An example of this is glycophorin A which forms a dimer. This dimer formation involves the precise packing of some of the amino acid side chains of the single transmembrane domain, as it can be abolished by even relatively conserved mutations of some of these side chains (Treutlein *et al.*, 1992). Another example is phospholamban, a cardiac ion channel, which can oligomerize forming up to a pentamer that is stable in SDS (Arkin *et al.*, 1994). We have described earlier that heating the AUM proteins can cause the uroplakins Ia and Ib to form large aggregates, although this process was difficult to control (Wu *et al.*, 1990). We found now, quite unexpectedly, that incubating the UPIa monomer in 1 M NH_2OH resulted in the formation of well defined UPIa dimers and trimers (Figs. 4 and 5). The propensity of UPIa, which may occupy the inner six domains of the 16-nm protein particle (see below), to oligomerize may play a role in AUM assembly.

The UPIb/UPIII Heterodimer—An interesting feature of UPIb and UPIII, that distinguishes them from UPIa and UPII, is that the former can be cross-linked much more efficiently than the latter. Thus 0.5–1 mM EGS, which barely cross-linked UPIa and UPII, yielded a nearly maximal amount of UPIb/UPIII heterodimer (Fig. 2, *c* and *d*). Increasing the EGS concentration to 2–5 mM led to the complete cross-linking of UPIb to form a homodimer and higher oligomers, the significant cross-linking of UPIII to form a homodimer, and the disappearance of the UPIb/UPIII heterodimer most likely due to its conversion to higher oligomers (Fig. 2). Although these cross-linked species had not been characterized as thoroughly as those involving UPIa and UPII, our data clearly showed that UPIb and UPIII, which could be cross-linked extremely efficiently to themselves and to each other, were not cross-linked to UPIa and UPII (Figs. 2–4). That UPIa and UPIb, two closely related members of the TM4 family, were cross-linked to different partners in AUMs raised the interesting possibility that they played different roles in AUM formation (see below).

Possible Heterogeneity of AUM Plaques

Perhaps one of the most intriguing aspects of AUM structure is: why do AUMs have two closely related UPIa and UPIb which share 39% of their amino acid sequences (Yu *et al.*, 1994), as well as two "type I proteins," the UPII and UPIII, which share a stretch of 12 amino acids located on the extracellular side of their single transmembrane domains (Wu and Sun,

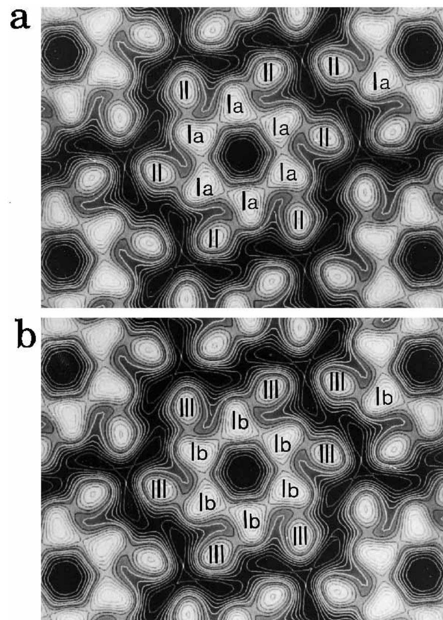

FIG. 8. **A schematic model showing the possible existence of two types of AUM plaques containing 16-nm particles that are composed of (*a*) uroplakins Ia and II and (*b*) uroplakins Ib and III.** *a*, in the UPIa/UPII model, the 27-kDa UPIa and the 15-kDa UPII are hypothesized to occupy the inner and outer domains of a 16-nm protein particle. This model can account for (i) the oligomerization of UPIa, (ii) the cross-linking of UPIa/UPII heterodimer, (iii) the cross-linking of UPII/UPII homodimer, and (iv) the selective disruption of the UPII homodimer formation by octyl glucoside. *b*, UPIb and UPIII occupy the inner and outer domains, respectively, of the UPIb/UPIII model. This model can account for the cross-linking of UPIb/UPIII heterodimer, as well as the efficient cross-linking of UPIb/UPIb and UPIII/UPIII homodimers. The stain-excluding map of bovine AUM was taken from Wu *et al.* (1994) (also see Walz *et al.* (1995)). For details, see the text.

1993; Lin *et al.*, 1994)? Another intriguing feature has to do with protein stoichiometry. Given the highly organized structure of AUM, one expects a precise and fixed stoichiometry of its protein subunits. Although as we have pointed out recently, the significantly different color yields of uroplakins when stained with Coomassie Blue and silver nitrate complicate their quantitation (Wu *et al.*, 1994), it now appears that the ratio of various uroplakins is quite variable.

With these questions in mind, it is interesting to note that the four uroplakins form two pairs as defined by the two known (cross-linked) heterodimers, *i.e.* UPIa/UPII and UPIb/UPIII. Each of these dimers consists of a four transmembrane-domained member (UPIa or UPIb) plus a single transmembrane-domained protein (UPII or UPIII). Although all of the uroplakins appear to be able to form oligomers (Figs. 2–5) (Wu and Sun, 1993; Wu *et al.*, 1994), so far we have not found conditions under which we can cross-link UPIa to UPIII (instead of UPII), or UPIb to UPII (instead of UPIII), suggesting a specificity in uroplakin interaction that was not suspected previously. This specificity raises the possibility that AUMs, despite the fact that they appear to be morphologically homogeneous, may actually contain two distinct populations of 16-nm protein particles, one composed of UPIa and UPII, and another of UPIb and UPIII (Fig. 8).

The UPIa/UPII Particle: A Model

As we and others have shown earlier, each 16-nm particle of the AUM can be resolved into 12 stain-excluding domains, that are arranged in an inner ring of six and outer ring of six (Hicks and Ketterer, 1969, 1970; Vergara *et al.*, 1969; Robertson and Vergara, 1980; Brisson and Wade, 1983; Walz *et al.*, 1995). Taylor and Robertson (1984) calculated that the volume of each inner domain is 1.6 times larger than that of an outer domain. If indeed there exist two types of 16-nm particles, one of them consisting of UPIa and UPII, it would be interesting to consider a model in which each (larger) inner domain contains a 27-kDa UPIa, while each (smaller) outer domain contains a 15-kDa UPII (Fig. 8*a*). Since this model depicts a central ring of six interconnected UPIa molecules (Fig. 8*a*), this attaches a possible significance to the observation that UPIa has a propensity to form dimers, trimers, and higher oligomers that are stable even in SDS (Fig. 5*a*, *horizontal arrows*). Since, as we have shown recently, each outer domain is connected via some fine "bridges" to an outer domain of a neighboring 16-nm particle (Walz *et al.*, 1995), this model predicts the cross-linking of UPIIs from two neighboring 16-nm particles resulting in the formation of dimers but no higher oligomers (Fig. 8*a*), which is indeed what we observed (Figs. 2–5). Also, since this kind of UPII dimer formation involves protein:protein interactions across two neighboring 16-nm particles (Fig. 8*a*), one may expect that this requires a longer armed cross-linking reagent and is more susceptible to detergent disruption than the formation of UPIa/UPII dimer which involves only intraparticle interactions; this is indeed what we observed (Figs. 6 and 7).

This kind of consideration also suggests that the UPIa/UPII particles are not intermixed, within a single plaque, with the other kind of UPIb/UPIII particles, because if that were the case we should see the cross-linking of UPII of one particle to the UPIII of a neighboring particle, and we have not yet seen that. This raises the possibility that there are two types of urothelial plaques, one consists purely of 16-nm particles containing UPIa and UPII, while the other consists of particles containing UPIb and UPIII. This hypothesis is schematically depicted in a working model, shown in Fig. 8, that can account for all of the available data. This model is attractive because it can solve two puzzles. It can explain the redundancy of uroplakins, as the two TM4 family members, *i.e.* the UPIa and Ib, may actually interact with different partners and thus play related but distinct roles in AUM formation. This hypothesis can also solve the stoichiometry puzzle, because it now predicts a molar relationship of UPIa = UPII and UPIb = UPIII, thus allowing variations in the overall stoichiometry, depending on the ratio of the two types of AUM plaques. In addition, this model predicts that the two types of AUMs may play different biological roles in terminally differentiated urothethelial cells. For example, since of all the known uroplakins only the UPIII has a long cytoplasmic domain, this uroplakin may play a role in anchoring the AUM plaques into a cytoskeletal network (Wu and Sun, 1993). Is it then possible that only the UPIb/UPIII plaques can bind to the cytoskeleton? Since uroplakin II is the only AUM protein that has a long preprosequence, we need to consider the possibility that the UPII prosequence may be involved in regulating AUM assembly in the Golgi (Lin *et al.*, 1994). Is it then possible that the assembly of the UPIa/UPII plaques is regulated differently from that of the other kind of plaques? Additional experiments are obviously needed to further study the possible heterogeneity of AUMs and to address some of the questions raised herein.

Complex Formation Involving Other TM4 Proteins

Our finding that UPIa, a TM4 protein, can form a specific complex with a small integral membrane protein, the UPII, is not unique among the TM4 proteins. For example, the 26-kDa TAPA-1 is known to interact with a 16-kDa Leu-13 antigen in leukocytes and activated endothelial cells (Takahashi *et al.*, 1990; Matsumoto *et al.*, 1993). In another instance, a 24-kDa CD9 has been shown to bind to a 14.5-kDa diphtheria toxin

receptor (DTR) which can also serve as the precursor of a heparin-binding EGF-like growth factor (HB-EGF/DTR) (Mitamura *et al.*, 1992; Brown *et al.*, 1993; Iwamoto *et al.*, 1994). In addition to enhancing the diphtheria toxin binding to its receptor, CD9 can potentiate the juxtacrine growth factor activity of membrane-bound HB-EGF/DTR (Higashiyama *et al.*, 1995). These results suggest that although TM4 proteins themselves lack an appreciable cytoplasmic domain, some of them can modulate the biological function of another integral membrane protein that has a cytoplasmic domain. Interestingly, the HB-EGF/DTR and UPII precursor, both of which bind to TM4 proteins, share some structural features (Naglich *et al.*, 1992; Lin *et al.*, 1994). Both have a cleavable signal peptide, followed by a prosequence that can potentially be removed by furin-like enzymes, both possess a single transmembrane domain located near the C terminus of the mature protein, and both are relatively basic (pro-UPII and HB-EGF have pI of 11.1 and 9.9, respectively). Whether these limited structural similarities are significant, or are merely coincidental, is currently unknown.

Imai and Yoshie (1993) have shown that CD81 and CD82, which coexist in T cells, can be coimmunoprecipitated, suggesting that they interact with each other forming a complex. Our finding that UPIa and UPIb, two members of the TM4 family, interact with different partners in AUM was therefore unexpected. Taken together, these data indicate that members of the TM4 family, although structurally related, have diverse structural and functional properties.

Concluding Remarks and Perspectives

In conclusion, we have shown that the four major structural proteins of AUM, *i.e.* uroplakins Ia, Ib, II, and III, can be divided into two pairs consisting of UPIa/UPII and UPIb/UPIII. We propose that these two uroplakin pairs can form two types of 16-nm protein particles that may assemble into plaques with different assembly and functional properties. This model can account for all the existing data, and it can explain several previously puzzling features of the AUM. Moreover, it makes specific predictions that can be tested experimentally. For example, it would now be interesting to determine whether antibodies monospecific for individual uroplakins (see, *e.g.* Fig. 1) would decorate only a subset of urothelial plaques, whether antibodies to UPIa and UPIb would preferentially associate with the inner domains while those against UPII and UPIII with the outer domains of some of the 16-nm AUM particles, and whether one can reconstitute the 16-nm protein particles with specific pairs of uroplakins (*e.g.* UPIa plus UPII). Regardless to what extent the current model will prove to be correct, these experiments should greatly advance our understanding on the structure and function of AUM, a fascinating and truly unique biomembrane.

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