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

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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-domain 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 distension, contains two TM4 members, the uroplakins (UPs) Ia and Ib. In association with two other (single transmembrane-domain) 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 UPIII 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) 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 SJ 23, 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 Yoshe, 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

* This work was funded in part by Veterans Administration Merit Review Award (to X.-R. W.) and by National Institutes of Health Grants DK47529, DK49469, and DK39753 (to T.-T. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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§ The abbreviations used are: TM4, transmembrane 4; EGS, ethylene glyco-bis(succinimidy l succinate); DFDNB, 1,5-difluoro-2,4-dinitrobenzene; PAGE, polyacrylamide gel electrophoresis; CHAPS, 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid; DTR, diphtheria toxin receptor; HB-EGF, heparin-binding epidermal growth factor.
with other integral membrane proteins?

Uroplakins Ia (UPIa; 27-kDa) and Ib (UPIb; 28-kDa; also known as T1-1) (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 plagues covering about 80% of the apical surface area of mammalian urothelium (Porter and Bonneville, 1963; Hicks, 1965; Porter et al., 1967; Koss, 1968; 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.

**Materials and Methods**

Isolation of Asymmetrical 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 ml Hepes/NaOH (pH 7.5) containing 1 ml each of phenylmethylsulfonyl fluoride, EDTA, and EGTA. After centrifugation at 2,000 × g for 10 min, the pellet was homogenized in the same buffer, transferred onto a 1.6 M sucrose cushion, and centrifuged at 100,000 × g for 20 min. The membranes located at the interface were collected, washed with 10 ml 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 ml Hepes buffer (pH 7.5) at room temperature for 10 min and recovered the (insoluble) asymmetrical unit membranes by centrifugation at 18,000 × g for 30 min (Wu et al., 1990, 1994). A portion of the AUMs was further treated with 25 ml NaOH, followed by washing with 10 ml 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 (DSNQGRELTRLWRC), uroplakin Ib (AKDDSSVRSFQGLLIFGNC), and uroplakin II (CDSGSGFTVRLSA and SAYQVTNLAPGTKYYI) and uroplakin III (CAHSHDQITGAEVPQK). The underlined, terminal cysteine residues were added so that these peptides could be conjugated to keyhole limpet hemocyanin or bovine serum albumin using m-maleimido-benzoyl-N-hydroxysuccinimide ester as a cross-linking agent. 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 Me2SO to make a 25-mg 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 ml Hepes/NaOH buffer (pH 7.5). After 2 h at room temperature, the reaction was quenched by adding 1 N TrisHCl (pH 7.4) to a final concentration of 50 mM. The cross-linked reaction mixtures were stored at 20℃ until further analysis.

Cleaveage 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, 19:1), 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 N NH4OH in 50 mM phosphate buffer (pH 8.5) at 37℃ for 2 h. The samples were then equilibrated in SDS sample buffer (50 mM TrisHCl, 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 gels were 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% NH4OH, 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 electroblottedly 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-
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Dctated 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 uroplakin (Fig. 1). An additional wash of these AUM preparations using EGS resulted in the formation of a 72-kDa band recognized by both anti-UPIb and anti-UPII; and a 48-kDa band recognized only by anti-UPII (labeled II/III). Also note in Panels c and d the formation of a 72-kDa band recognized by both anti-UPIa 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. 1a, 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. 1a, lanes 2 and 3). Immunoblotting of these purified AUM preparations revealed a 48-kDa, UPIa-related protein (Fig. 4a, 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/UPIII heterodimer, and a 48-kDa UPIa/UPIII homodimer (Fig. 4), thus confirming and extending some of the crude membrane data (Figs. 2 and 3).

**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 (AgNO₃) 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).

**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-UPIII, (c) anti-UPIb, and (d) anti-UPIIII.
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 1M NH₂OH, 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. 5a). 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 homodimer (Fig. 5; dotted line). Finally, we observed a series of UPIa oligomers of 48 kDa (dimer) and 70 kDa (trimer), which were almost no effect on the formation of the UPIa/UPII heterodimer (Fig. 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 e-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/UPII homodimer. However, it failed to cross-link the UPII/UPII dimer (Fig. 6c), suggesting that the e-lysines in UPII/UPII cross-linking were >3 Å apart.

**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 UPII/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 a few major protein subunits (Wu et al., 1990, 1994; Yu et al., 1994). It was unexpected, however, that crude urothelial mem-

**Table I**

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<th>Uroplakin</th>
<th>SDS-PAGE</th>
<th>Tricine-SDS-PAGE*</th>
<th>cDNA-deduced</th>
<th>Sugar</th>
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<tr>
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<td>Nonreduced</td>
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<td>GS</td>
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<td>Ia</td>
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<td>47</td>
<td>47</td>
<td>18</td>
<td>72</td>
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a Unpublished data.  
b Yu et al. (1994).  
c ND, not detectable.  
Lin et al. (1994).  
Wu et al. (1993).
Incubated with only a buffer (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-UPI and with (b) anti-UPII. Note the generation of the UPII/UPII homodimer, the UPIa/UPII heterodimer, and a UPIa/UPII homodimer, thus confirming the crude membrane results. A 65-kDa species (?) may represent a UPIa oligomer (see Fig. 5a and text).

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 UP Ib, 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 yielding exclusively a monomeric 15-kDa 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/UP II 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/UP II 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 3 Å (versus 16 Å of EGS; Fig. 6c), suggesting a close proximity of the cross-linked ε-amino groups. On the other hand, the hydrophilic analogue of EGS, i.e. the sulfoglycolyl 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 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 sulfoglycolyl 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. 6c). 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. 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-UPI and with (b) anti-UPII. Note the generation of the UPII/UPII homodimer, the UPIa/UPII heterodimer, and a UPIa/UPII homodimer, thus confirming the crude membrane results. A 65-kDa species (?) may represent a UPIa oligomer (see Fig. 5a and text).

**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 N 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 (AgNO3), (b) immunoblotted with anti-UPIa and (c) immunoblotted with 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, b, and c, represent oligomerized UPIa (see text).
Although it yielded the UPIa/UPII heterodimer. The relative positions of uroplakin monomer and dimer are indicated on the X-100 (as well defined oligomers during SDS-PAGE (Lemmon et al., 1992; Treutlein et al., 1992; Arkin et al., 1994). An example of this is glycoporphin 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 NH₂OH 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 UPII 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, 1992; Treutlein et al., 1992; Arkin et al., 1994). An example of this is glycoporphin 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 NH₂OH 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).

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The four uroplakins form two pairs as defined by the two known ratio of various uroplakins is quite variable. The UPIII (Fig. 8).

cles, one composed of UPIa and UPII, and another of UPIb and UPIII. 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.

Selective Interactions of UPIa and UPIb

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/UPIII and UPIb/UPIII. Each of these dimers consists of a four transmembrane-domain member (UPIa or UPIb) plus a single transmembrane-domain 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 UPIII (instead of UPII), 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 particle interactions; this is indeed what we observed (Figs. 6 and 7).

This kind of consideration also suggests that the UPIa/UPIII 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 UPIII 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 = UPIII 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 urothelial 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 presequence may be involved in regulating AUM assembly in the Golgi (Lin et al., 1994). Is it then possible that the assembly of the UPIa/UPIII 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 hepatitis-B-envelope-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 juxtaorgan 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 respectively). Whether these limited similarities are truly coincidental, or are merely coincidental, is currently unknown.

Imai and Yoshie (1993) have shown that CD81 and CD82, which exist in T cells, can be immunoprecipitated, 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 may specify 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 UPIII 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. UPII plus UPIII). 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.

Acknowledgments—We thank Drs. Herbert Lepor and Irwin M. Freedberg for their continued interest in and support of this project.