Uroplakins Ia and Ib, Two Major Differentiation Products of Bladder Epithelium, Belong to a Family of Four Transmembrane Domain (4TM) Proteins

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Abstract. The mammalian bladder epithelium elaborates, as a terminal differentiation product, a specialized plasma membrane called asymmetric unit membrane (AUM) which is believed to play a role in strengthening and stabilizing the urothelial apical surface through its interactions with an underlying cytoskeleton. Previous studies indicate that the outer leaflet of AUM is composed of crystalline patches of 12-nm protein particles, and that bovine AUMs contain three major proteins: the 27- to 28-kD uroplakin I, the 15-kD uroplakin II and the 47-kD uroplakin III. As a step towards elucidating the AUM structure and function, we have cloned the cDNAs of bovine uroplakin I (UPI). Our results established the existence of two isoforms of bovine uroplakin I: a 27-kD uroplakin Ia and a 28-kD uroplakin Ib. These two glycoproteins are closely related with 39% identity in their amino acid sequences. Hydropathy plot revealed that both have four potential transmembrane domains (TMDs) with connecting loops of similar length. Proteolytic digestion of UPIa inserted in vitro into microsomal vesicles suggested that its two main hydrophilic loops are exposed to the luminal space, possibly involved in interacting with the luminal domains of other uroplakins to form the 12-nm protein particles. The larger loop connecting TMD3 and TMD4 of both UPIa and UPIb contains six highly conserved cysteine residues; at least one centrally located cysteine doublet in UPIa is involved in forming intramolecular disulfide bridges. The sequences of UPIa and UPIb (the latter is almost identical to a hypothetical, TGFβ-inducible, TI-1 protein of mink lung epithelial cells) are homologous to members of a recently described family all possessing four transmembrane domains (the "4TM family"); members of this family include many important leukocyte differentiation markers such as CD9, CD37, CD53, and CD63. The tissue-specific and differentiation-dependent expression as well as the naturally occurring crystalline state of uroplakin I molecules make them uniquely suitable, as prototype members of the 4TM family, for studying the structure and function of these integral membrane proteins.

T HE asymmetric unit membrane (AUM)[1] is a unique plasma membrane elaborated as a major differentiation product of the mammalian urothelium which lines the surfaces of renal pelvis, ureter, bladder, and urethra (Porter and Bonneville, 1963; Porter et al., 1967; Hicks, 1975). It forms numerous plaques covering the apical surface of the superficial cells of the multi-layered urothelium. The name AUM reflects the fact that its outer leaflet is almost twice as thick as its inner one (Hicks, 1965; Koss, 1969; Pauli et al., 1983). The thickened outer leaflet is composed of crystalline patches of 12-nm particles, each of which consists of six inner and six outer domains (Hicks and Ketterer, 1969; Vergara et al., 1969; Hicks and Ketterer, 1970; Knutton and Robertson, 1976; Robertson and Vergara, 1980). Morphological and physiological studies suggest that AUM may be involved in strengthening and stabilizing the urothelial cell surface, possibly through its interactions with an underlying cytoskeleton, thus preventing the cells from rupturing during bladder distention (Staehelin et al., 1972; Minsky and Chlapowski, 1978; Lewis and deMoura, 1984; Sarikas and Chlapowski, 1986). We have recently demonstrated that highly purified bovine AUM plaques, when analyzed by SDS-PAGE, yielded three major protein bands in the molecular weight ranges of 47, 27 to 28, and 15 kD (Wu et al., 1990). Antibodies have been raised against these proteins

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1. *Abbreviations used in this paper*: AUM, asymmetric unit membrane; CNBr, cyanogen bromide; 4TM, four transmembrane domain; KLH, keyhole limpet hemocyanin; MBS, m-maleimidobenzol-*N*-hydroxysuccinimide; TMD, transmembrane domains; UPI-III, uroplakin I-III.

and used to demonstrate by immunocytochemistry at the ultrastructural level that they are all AUM-associated in situ (Wu et al., 1990). Based on their established association with the AUM of urothelial plaques, we named these proteins uroplakin I (27 to 28 kD), uroplakin II (15 kD), and uroplakin III (47 kD).

In this paper, we have chosen to study the 27- to 28-kD uroplakin I (UPI) which has been identified as a major protein component of not only bovine AUMs, but also AUMs of pig, sheep, and rat (Ketterer et al., 1973; Vergara et al., 1974; Caruthers and Bonneville, 1977; Stubbs et al., 1979; Wu et al., 1994). We have previously raised a monoclonal antibody (AE31) and several polyclonal antisera to this component. The AE31 monoclonal antibody recognizes a 27-kD protein which was shown by EM to be associated with the outer leaflet of urothelial apical AUMs (Yu et al., 1990). Immuno-affinity chromatography using this antibody yielded, in one step, the corresponding 27-kD antigen, which co-purified with UPII and UPIII most likely through specific protein interactions (Yu et al., 1990). In an independent experiment, we generated antisera against all the uroplakins by immunizing rabbits with native bovine AUMs. Antibodies to UPI were affinity-purified from these anti-AUM antisera using the electrophoretically purified 27- to 28-kD component. These affinity-purified antibodies recognized specifically a 28-kD protein band by immunoblotting, and again decorated the outer leaflet of apical AUMs (Wu et al., 1990; 1994). However, these polyclonal antibodies failed to recognize the AE31-purified 27-kD component (Wu, X.-R., unpublished observation). Moreover, we have recently found that although the 27 to 28-kD component of bovine AUMs appear as a broad band on SDS-PAGE, that of the rabbit AUMs can be resolved into two well-defined bands (Wu et al., 1994). Taken together, these data strongly suggest that the 27- to 28-kD component of bovine AUMs, previously thought to consist of only one protein, is heterogeneous.

To address the issue of heterogeneity and to enhance our understanding on how this major 27- to 28-kD AUM component may participate in the formation of the 12-nm protein particles, we have set out to clone its cDNA. We describe here the cloning and characterization of two types of bovine UPI cDNAs, one encoding Uroplakin Ia—a 27-kD glycoprotein, and another encoding uroplakin Ib—a 28-kD glycoprotein. These two proteins are 39% identical in their amino acid sequences and both contain four potential transmembrane domains (TMDs). Studies on the proteolytic digestion of uroplakin Ia inserted in vitro into dog pancreatic microsomes suggest that its two hydrophilic loops interconnecting TMDs 1/2 and TMDs 3/4 are exposed to the luminal (exoplasmic; exo) space possibly involved in interacting with the luminal portions of other uroplakins to form the 12-nm protein particles. Very little cytoplasmic (cyto) domains exist for UPIa and Ib. Thus, like UPII and III, the two uroplakin I molecules have an extremely high ratio of $Mass_{exo}/Mass_{cyto}$. Such a highly asymmetric mass distribution of all the major AUM protein subunits may contribute to the thickened appearance of the outer leaflet of AUM. Finally, we present evidence that uroplakins Ia and Ib share significant sequence homology with a recently described family of membrane proteins called the "four transmembrane domain" (4TM) family. Members of this gene family include several important leukocyte differentiation markers such as CD9,

CD37, CD53, and CD63. The tissue-specific and differentiation-dependent expression and the naturally occurring crystalline state of uroplakins Ia and Ib make them ideally suitable, as prototype members of this family, for studying the expression, structure and function of these unique plasma membrane molecules.

Materials and Methods

Amino Acid Sequencing

Bovine AUMs were purified by sucrose density gradient centrifugation as described (Wu et al., 1990). The AUM protein components (without prior reduction) were separated by SDS-PAGE (15% acrylamide), and then transferred electrophoretically to Immobilon-PVDF membrane (Millipore, Bedford, MA) and stained with Coomassie blue. The 27- to 28-kD protein band corresponding to uroplakin I was excised and subjected to NH_2-terminal amino acid sequencing. For generating internal sequences, the electrophoretically purified 27- to 28-kD protein was digested by cyanogen bromide (CNBr) or trypsin (Lin et al., 1994). The CNBr–peptide fragments were resolved by SDS-PAGE (16.5% acrylamide, 6% bisacrylamide) in a Tricine buffer (Schagger and von Jagow, 1987), while the tryptic peptides were fractionated by reverse phase HPLC. The NH_2-terminal sequences of all the major, well-resolved peptide bands or peaks were determined. Automatic amino acid sequencing was performed using an ABI model 477A protein sequencer (courtesy of William Lane, Harvard Microchemistry Facility, Cambridge, MA).

Polymerase Chain Reaction and Screening of cDNA Library

Total RNAs were isolated form bovine tissues and cultured bovine urothelial cells (Sambrook et al., 1989), and poly(A)+ mRNAs were purified using an oligo-dT cellulose column (type 3; Collaborative Research, Boston, MA). Single-stranded cDNAs of these mRNA were synthesized using avian myoblastosis virus reverse transcriptase (Promega, Madison, WI) and oligo-dT primers. Degenerate oligonucleotide primers in both sense and antisense orientations were synthesized based on three partial amino acid sequences: PEVVFP, MLTFYS, and DYLFTK as indicated in Fig. 2. To facilitate the subcloning of the PCR products, additional HindIII and EcoRI linkers were placed on the 5'-end of the sense and antisense primers, respectively. PCRs were performed with Taq polymerase (Perkin-Elmer Cetus, Norwalk, CT) using bovine liver or bladder epithelial single-stranded cDNA as the template. 30 thermal cycles were performed, each consisting of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min. The PCR products were resolved in a 1.5% agarose gel. Selected bands were eluted, subcloned into pGEM-72 (Promega Biotec, Madison, WI) and sequenced using a T7 DNA Sequencing Kit (Version 2; US Biochemical, Cleveland, OH). Specific PCR products were ^{32}P-labeled and used to screen a λgt10 cDNA library of bovine bladder epithelium (Lin et al., 1994). Hybridization was carried out at 60°C overnight in a solution containing 6× SSC, 5× Denhardt's solution, 1% SDS, and 100 μg/ml of denatured salmon sperm DNA. cDNA inserts from positive phage were subcloned into pGEM7Z and sequenced. The immunological screening of a λgt11 expression library was done according to standard methods (Sambrook et al., 1989). The cDNA inserts of positive clones were subcloned into pGEM3Z and sequenced in both strands.

Northern Blot Analysis

20 μg of total cellular RNAs were fractionated on a 1% agarose gel containing formamide, and transferred onto a nylon filter (Hybond; Amersham Corp., Arlington Heights, IL). The blot was hybridized with ^{32}P-labeled UPIa or UPIb cDNA at 42°C overnight in 6× SCC, 50% formamide, 1× Denhardt's solution, 0.1% SDS, 10% dextran, 0.1 M EDTA, and 50 μg/ml denatured salmon sperm DNA. The filter was then washed at 65°C in 0.2× SCC and 0.1% SDS for 30 min. In some cases, the probe was stripped off by washing the filter in 50% formamide and 2× SCC at 80°C for 1 h followed by rehybridizing with a ^{32}P-labeled human β-actin cDNA.

Primer Extension Assay

Primer extension was carried out using a kit from Promega Biotec. Three

oligonucleotide primers, P1 (25 mer), P2 (25 mer), and P3 (17 mer), were synthesized based on nucleotide sequences complementary to nucleotides 195–219, 102–126, and 75–91 of UPIa cDNA, respectively (see Fig. 4). A ^{32}P-labeled primer (0.5 pmole) was annealed with 10 μg of total RNA from bovine liver, bladder epithelium or cultured urothelial cells, and reverse transcribed at 42°C for 30 min. The extended products were then analyzed on an 8% DNA sequencing gel. The size ladder was generated from a control M13 template and a forward primer using a T7 Sequenase Kit.

Generation of Antibodies Against Synthetic Peptides

Two peptides, "DSNQGRELTRLWDR" and "AKDDSTVRSFQGLLIFGN," were chemically synthesized (courtesy of Dr. William Lane) based on the cDNA-derived amino acid sequences of UPIa and UPIb, respectively (Figs. 4 and 5). An additional cysteine residue was placed at the COOH terminus of each peptide to facilitate the conjugation to the carrier proteins. The underlined serine residue of the UPIb peptide replaced an original cysteine to avoid excessive cross-linking. These two peptides were crosslinked to keyhole limpet hemocyanin (KLH) or bovine serum albumin using m-male-imidobenzol-N-hydroxysuccinimide (MBS). 100 μg of the conjugated peptide were used to immunize each rabbit for the primary injection, and 50 μg for booster injections at 2-wk intervals.

In Vitro Translation and Microsome Insertion Assay

The UPIa cDNA, subcloned in pGEM7Z, was used to transcribe in vitro capped mRNA using a Riboprobe Kit (Promega Biotec) and m7G(5')ppp (5')G (Pharmacia Fine Chemicals, Piscataway, NJ). Translation in the presence and absence of dog pancreatic microsomes was performed using [^{35}S]methionine in a rabbit reticulocyte lysate system (Promega Biotec) as described (Lin et al., 1994). For deglycosylation assays, the translation products were boiled in 1% SDS for 3 min, and then incubated with 100 mU/ml of endoglycosidase H (Boehringer Mannheim Biochemicals, Indianapolis, IN) in 150 mM sodium citrate (pH 6.0), 0.2% SDS, and 2 mM PMSF at 37°C overnight. For protease protection assays, a mixture of trypsin and chymotrypsin was added to a final concentration of 100 μg/ml. The digestion was performed on ice for 1 h and terminated by adding a protease inhibitor, leupeptin. In some experiments, Triton X-100 was added to a final concentration of 0.5% to solubilize the microsome vesicles before protease digestion. All the above translation products were analyzed by SDS-PAGE on a 15% gel and autoradiographed.

Deglycosylation and Western Blot Analysis

Bovine AUMs were solubilized in 0.5% SDS at room temperature for 15 min. The membrane mixtures were then incubated with endoglycosidase H in a citrate buffer (150 mM sodium citrate, pH 6.0, 0.2% SDS, 1% octyl glucoside and 2 mM PMSF) or with N-glycosidase F (Boehringer Mannheim Biochemicals) in a phosphate buffer (50 mM sodium phosphate, pH 7.5, 0.1% SDS, 1% octyl glucoside, and 2 mM PMSF). After incubation at 37°C for 16 h, the reaction mixtures were resolved by SDS-PAGE and electrophoretically blotted onto a nitrocellulose filter. In some cases, proteins were reduced with 5% β-mercaptoethanol before loading onto the gel. UPIa and UPIb were detected by Western blot analysis (Eichner et al., 1984) using antisera against synthetic peptides unique to each UPI.

Sequence Analysis

The nucleotide and the deduced amino acid sequences of UPIa and UPIb were compared with the entries in GenBank, EMBL, PIR-Protein, and SwissProt data bases through FASTA or TFASTA algorithms (GCG7 package; University of Wisconsin, Madison, WI). The secondary structures of proteins were predicted by the PEPTIDESTRUCTURE program (GCG7 package) or GeneWorks 2.0 (IntelliGenetics, Inc., Mountain View, CA). Multiple sequence alignment was carried out using the LINEUP and PILEUP programs (GCG7 package).

Results

Isolation of cDNAs Encoding Two Closely Related Uroplakin I Molecules

Taking advantage of the extreme insolubility of AUMs in a broad spectrum of detergents, we have previously devised a way for isolating large amounts of bovine AUMs using sucrose density gradient centrifugation followed by dissolving the non-AUM contaminating membranes in deoxycholate (Wu et al., 1990). Such highly purified AUMs are morphologically intact exhibiting crystalline patches of 12-nm protein particles, which contain three major protein bands when analyzed by SDS-PAGE (Fig. 1 a, lane 2). We sequenced the NH₂ terminus of the 27- to 28-kD protein and obtained a mixture of two sequences (Fig. 2). To generate additional, internal sequences, we digested the electrophoretically purified 27- to 28-kD uroplakin I protein (Fig. 1 a, lane 3) with
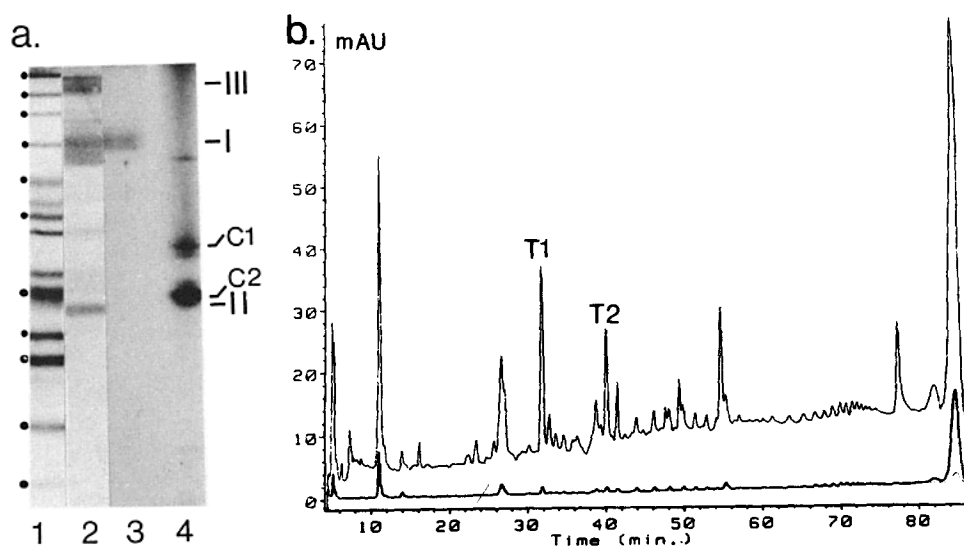
Figure 1. Isolation of CNBr and tryptic peptides of bovine 27- to 28-kD uroplakin I. (a) Electrophoretic separation of bovine uroplakins. AUMs were purified from bovine bladder epithelium and the three major uroplakins (I, II, and III) were resolved, without prior reduction, by preparative Tricine/SDS-PAGE (lane 2). The protein markers (lane 1) have molecular masses of 66, 45, 36, 29, 24, 20, 17, 14, 8, 6.2, and 2.5 kD (dotted from top to bottom). The 27- to 28-kD UPI band was electrophoretically purified (lane 3), cleaved with cyanogen bromide (CNBr), and again resolved electrophoretically without prior reduction (lane 4). Note the clear resolution of two major CNBr fragments C1 and C2 (lane 4). (b) Tryptic peptides. The 27- to 28-kD UPI protein was electrophoretically transferred from a preparative SDS polyacrylamide gel onto a nitrocellulose filter, and digested in situ with trypsin. The released peptide fragments were separated by reverse phase HPLC, and the NH₂-terminal sequences of the peptides of the two major peaks (T1 and T2) were determined.

A. Primary Data:

(1) C1   (D/L)-(T/E)-(F/V)-Y-(S/F)-(T/A)-(S/D)-(A/S)-(F/N)-(R/Q)-(A/G)-(T/I)-

(2) C2   (I/D)-(E/W)-(Q/V)-E-F-T-(S/G)-A-F-R-A-T-T-P-E-V-V-F-P-

(3) T1   (D/Q)-(M/Y)-(L/M)-(V/T)-(F/S)-(Y/N)-(S/P)-(A/S)-(D/L)-(S/I)-(N/T)-
          (Q/K)-G-

(4) T2   (Q/L)-(M/G)-(L/H)-(T/L)-(F/D)-Y-L-F-T-K

(5) NH₂   (D/A)-(K/F)-D-(D/I)-S-(W/T)-(V/L)-(R/S)-G-(L/F)-(L/Q)-(S/Q)-(P/L)-
         (V/L)-(M/I)-(F/T)-(G/E)-(N/L)-V-(I/L)-

## B. Deduced Sequences:

#1 C1 and C2   D-(E)-V-?-F-T-S-A-F-R-A-T-T-P-E-V-V-F-P-

#2 C1 only   L-T-F-Y-S-A-D-S-N-Q-G-I-

#3 C2 only   I-(W)-Q-E-?-?-G-

#4 T1, T2 and #2   Q-M-L-T-F-Y-S-A-D-S-N-Q-G-

#5 T1 only   D-Y-M-V-S-N-P-S-L-I-T-K

#6 T2 only   L-G-H-L-D-Y-L-F-T-K

Figure 2. Partial amino acid sequences of bovine 27- to 28-kD uroplakin I. (*A*) Primary data showing the original partial amino acid sequences generated by automatic sequencer. C1 and C2 are the NH₂-terminal sequences of two CNBr-peptides (Fig. 1 *a*); T1 and T2 are NH₂-terminal sequences of two tryptic peptides (Fig. 1 *b*); while NH₂ represents the NH₂-terminal sequences of electrophoretically purified, intact 27- to 28-kD protein. All amino acids are represented by single letter codes. In many cases two amino acids were identified from one cycle of Edman sequencing; these two amino acids are listed in a parenthesis. (*B*) The amino acid residues shared by C1 and C2 peptides (plus the remaining COOH-terminal C2 sequence) are shown as "#1" sequence. Subtraction of this sequence from C1 and C2 sequences yielded the "#2" and "#3" sequence, respectively. Amino acids shared by T1 and T2 (as well as the "#2" sequence) are listed as "#4" sequence. Subtraction of this sequence from T1 and T2 sequences gave rise to "#5" and "#6" sequences, respectively. The two amino acid assignments in parentheses were ambiguous and were later found to be erroneous according to cDNA data. "?" indicates uncertainty. From the three underlined sequences (*1* to *3*), degenerate oligonucleotide primers were synthesize in both sense and antisense directions and were used to amplify the cDNAs of UPI by PCR.

cyanogen bromide yielding two major peptides (C1 and C2; Fig. 1 *a*, lane *4*). In addition, we trypsinized UPI and isolated two major tryptic peptides of uroplakin I by reverse phase HPLC (T1 and T2; Fig. 1 *b*). The NH₂-terminal sequences of all the above peptides, most of them containing a mixture of two sequences, are listed in Figure 2 *A*. Based on the fact that C1 and C2 sequences contain an overlapping sequence D-(E)-V-?-F-T-etc (Fig. 2 *B*, sequence *1*), we deduced two other sequences by subtraction (Fig. 2 *B*, sequences *2* and *3*). Similarly, from the two "double" sequences of T1 and T2 we were able to deduce three sequences (Fig. 2 *B*, sequences *4–6*).

Based on some of these amino acid sequences, we designed three degenerate oligonucleotide primers to perform PCR using the cDNAs of bovine bladder epithelium and liver as the templates (Fig. 3 *a*, and data not shown). Only one particular combination of these primers (2→3) yielded a bladder-specific PCR product of about 270 bp in length (Fig. 3 *a*, lane *1*). When this product was cloned and used as a probe for Northern blot analysis, a 1.5-kb mRNA was detected in bovine bladder (Fig. 3 *b*, lane *1*) but not in the liver (lane *2*). Using this partial cDNA as a probe, we screened a bovine urothelial cDNA library and obtained eight clones containing overlapping cDNAs. The combined nucleotide sequence of these clones and its corresponding amino acid sequence are shown in Fig. 4. This cDNA sequence (1,363-bp
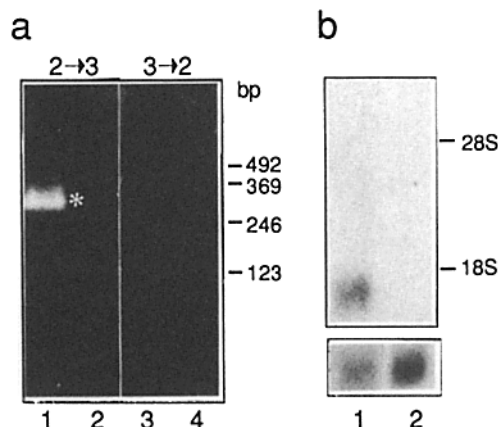
Figure 3. Generation of a sequence- and urothelium-specific partial DNA of bovine uroplakin I (UPI) by PCR. (*a*) PCRs were performed using different combinations of oligonucleotide primers (marked *1* to *3*, Fig. 2 *B*) in both directions. The templates were cDNA of either bovine bladder epithelium (lane *1* and *3*) or liver (lane *2* and *4*), and the products were resolved electrophoretically and stained with ethidium bromide. Only one combination of primers consisting of sense primer 2 and antisense primer 3 ("2→3") yielded a urothelium-specific PCR product of ∼270 bp (*asterisk*; *a*, lane *1*). The upper part of *b* is a Northern blot showing that this 270-bp bladder-specific PCR product detected a 1.5-kb mRNA in bovine urothelium (lane *1*) but not in liver (lane *2*); the lower part shows the detection of β-actin mRNA as an internal control.

long) contains a short 5'-untranslated sequence of 18 bp, a single open reading frame of 774 bp, followed by a 571 bp of 3'-untranslated region containing a polyadenylation signal. The open reading frame encodes a polypeptide of 258 amino acids which can account for all six partial, internal amino acid sequences of the 27- to 28-kD uroplakin I protein listed in Fig. 2 *B*. However, it cannot account for the two NH₂-terminal sequences. Although the predicted initiator methionine is in a context favorable for translation initiation (Kozak, 1984) and the length of the cDNA is close to that of the corresponding mRNA (Fig. 3 *b*), we performed several experiments to test the possibility of an incomplete 5' sequence. Primer extension assays were done using an independent batch of bovine urothelial cDNA as the template. Three different primers (see Materials and Methods) yielded extension products terminating at the same 5' end of the existing cDNA (data not shown). Similarly, using the 5' RACE (rapid amplification of cDNA end) approach (Frohman et al., 1988) we failed to generate additional sequences beyond the 5' end of our current cDNA clones. These results strongly suggest that the unaccounted for NH₂ terminal sequences of the intact 27- to 28-kD uroplakin I are due to other proteins.

To clone these other uroplakin I molecules, we tried several approaches including the immunoscreening of a cDNA expression library. We have previously immunized rabbits with intact bovine AUMs containing all uroplakins (see Introduction). Antibodies to the 27- to 28-kD uroplakin I were affinity purified and used to screen a λgt11 expression library of bovine urothelial cells. An ensuing positive clone was used to re-screen the library. Several partially overlapping clones were obtained, and their combined nucleotide sequence along with its theoretically translated amino acid se-
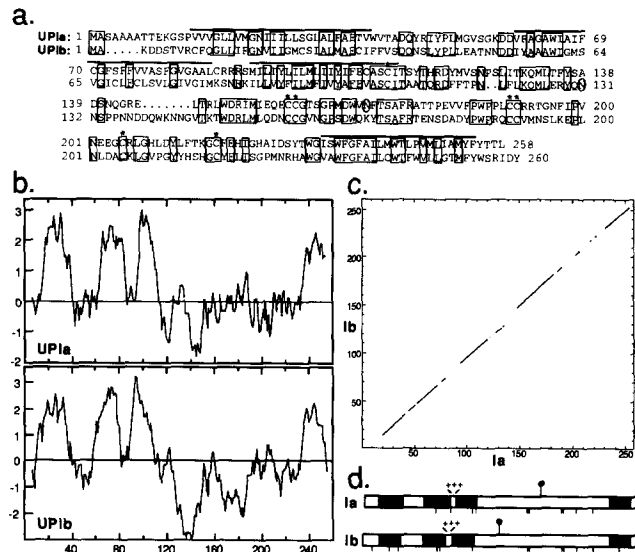
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                                              GGCAGAGAAGGCAGGACT
19 ATGGCTTCTGCAGCAGCAGCAACGACAGAGAAGGGGTCTCCAGTTGTGGTGGGTCTGCTGGTCATGGGCAACATC
1  M  A  S  A  A  A  A  T  T  E  K  G  S  P  V  V  V  G  L  L  V  M  G  N  I

94 ATTATTCTGCTGTCAGGCCTGGCCCTGTTTGCTGAAACGGTATGGGTGACCGCTGACCAGTACCGCATATACCCG
26 I  I  L  L  S  G  L  A  L  F  A  E  T  V  W  V  T  A  D  Q  Y  R  I  Y  P

169 CTGATGGGCGTCTCGGGCAAGGATGACGTCTTCGCCGGCGCCTGGATCGCCATCTTCTGCGGCTTCTCCTTCTTC
51  L  M  G  V  S  G  K  D  D  V  F  A  G  A  W  I  A  I  F  C  G  F  S  F  F

244 GTGGTGGCCAGCTTTGGTGTGGGCGCAGCACTCTGCCGCCGCCGCTCCATGATCCTCACGTACCTGATACTCATG
76  V  V  A  S  F  G  V  G  A  A  L  C  R  R  R  S  M  I  L  T  Y  L  I  L  M

319 CTCATCATCTACATCTTTGAGTGCGCCTCCTGCATCACGTCCTACACCCACCGAGACTATATGGTGTCCAACCCG
101 L  I  I  Y  I  F  E  C  A  S  C  I  T  S  Y  T  H  R  D  Y  M  V  S  N  P

394 TCCCTGATCACCAAGCAGATGTTGACATTCTATAGTGCAGACTCGAACCAGGGCCGGGAACTGACCCGCCTCTGG
126 S  L  I  T  K  Q  M  L  T  F  Y  S  A  D  S  N  Q  G  R  E  L  T  R  L  W

469 GATCGCATCATGATTGAGCAAGAGTGCTGTGGCACGTCAGGCCCCATGGACTGGGTGAACTTCACGTCTGCCTTC
151 D  R  I  M  I  E  Q  E  C  C  G  T  S  G  P  M  D  W  V  (N) F  T  S  A  F

544 CGGGCCACCACCCCAGAGGTGGTGTTCCCCTGGCCCCCGCTATGCTGTCGACGGACCGGCAACTTCATCCCAGTC
176 R  A  T  T  P  E  V  V  F  P  W  P  P  L  C  C  R  R  T  G  N  F  I  P  V

619 AATGAAGAAGGCTGCCGCCTGGGCCACCTGGACTACCTGTTCACCAAGGGCTGCTTTGAACATATTGGCCACGCC
201 N  E  E  G  C  R  L  G  H  L  D  Y  L  F  T  K  G  C  F  E  H  I  G  H  A

694 ATCGACAGCTACACGTGGGGCATCTCGTGGTTTGGGTTTGCCATCCTGATGTGGACGCTCCCCGTGATGCTGATA
226 I  D  S  Y  T  W  G  I  S  W  F  G  F  A  I  L  M  W  T  L  P  V  M  L  I

769 GCCATGTATTTCTACACCACGTTGTGAGAACGAGAAGTGAAGGCCACGTGCACACCTGGCTTCCTCCTCCTCCTG
251 A  H  Y  F  Y  T  T  L  *

844 CTCTGGCTTCCTCTGGCTGAGATGGCCGACTCGCCTCTCCCTGTCCCACCTCCCTGGCCCAGTCCTCCCTCCACT
919 CCAAAGATGTTTTACCAGGTTTCTGAGCCCTGCTGAGAGTCGGGGTGCCCTAAAACCCCTGGACATCCTCCTTACT
994 AACGACTAAGCTTCCAGCAAATTCTCTAAGGGGTGTTAGCATGTGTGTACAGACCGTTAGTCCTTAACCTCCTT
1069 TCACTAGACTGATTCTTGGCCCATCTTTCAGGGTCAACTTCAAGTCCTGTCCTCGGGGGGGCCCTTTCCTGATCT
1144 CACCACCCCATTCACAGATGCCTTTCTTATAGTTCCCAGAGCTCCTCCTCCATGGTGGATGTCATCATCATCATGT
1219 GAATAGTTTGTGATTGTCTGTTTAAATTCTGGTAGAACTGGGATTGCCATGAGGGAGAGGGACAAGTTCTGTTATG
1294 GTCACTTTAACATCCCTGCATCACCTGGCATGGGGCTGAGCACGGACATTCAATAAATACTACTTGAATG(A)n
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Figure 4. The deoxynucleotide and deduced amino acid sequence of bovine uroplakin Ia (UPIa). The stretches of deduced amino acid sequences which match with protein microsequencing data are underlined and numbered according to Fig. 2 *B*; inconsistencies are underlined by dashes. Four stretches of hydrophobic amino acids that are long enough to span the lipid bilayer are boxed and shaded. A potential N-linked glycosylation site is circled, and the polyadenylation signal near the poly(A) tail is underlined by a thick bar.

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1 GCGTGCAGAGAGCCGCACACAGTACCCAGGAGGAGGAGGAGAGGCTTGGGGGAAATCCTGAAG
62 ATGGCCAAAGACGACTCCACTGTTCGTTGCTTCCAGGGCCTGCTGATTTTTGGAAATGTGATTATCGGTATGTGC
1  M  A  K  D  D  S  T  V  R  C  F  Q  G  L  L  I  F  G  N  V  I  I  G  M  C

137 AGCATCGCCCTGATGGCAGAGTGCATCTTCTTTGTATCAGACCAAAACAGCCTCTACCCACTGCTTGAAGCCACC
26  S  I  A  L  M  A  E  C  I  F  F  V  S  D  Q  N  S  L  Y  P  L  L  E  A  T

212 AACAATGACGACATCTATGCGGCAGCCTGGATTGGCATGTCTGTTGGCATCTGCCTCTTCTGCCTCTCTGTCCTG
51  N  N  D  D  I  Y  A  A  A  W  I  G  M  S  V  G  I  C  L  F  C  S  L  V  L

287 GGCATCGTAGGCATCATGAAGTCCAACAGGAAAATTCTTCTGGTGTATTTCATCCTGATGTTTATTGTATATGCT
76  G  I  V  G  I  M  K  S  N  R  K  I  L  L  V  Y  F  I  L  M  F  I  V  Y  A

362 TTTGAAGTGGCATCTTGTATCACAGCAGCAACACAACGAGACTTTTCCTACACCCAACCTCTTCCTGAAGCAGATG
101 F  E  V  A  S  C  I  T  A  A  T  Q  R  D  F  F  T  P  N  L  F  L  K  Q  M

437 CTGGAGAGGATACCAAAACAACAGTCCTCCAAACAATGATGACCAATGGAAAAACAATGGAGTCACCAAGACCTGG
126 L  E  R  Y  Q  (N) S  P  P  N  N  D  D  Q  W  K  N  N  G  V  T  K  T  W

512 GACAGACTTATGCTCCAGGACAATTGCTGTGGTGGTAAATGGCCCGTCAGACTGGCAAAATACACCTCTGCCTTC
151 D  R  L  M  L  Q  D  N  C  C  G  V  K  W  P  V  R  L  A  K  Y  T  S  A  F

587 CGGGACTGAGAACAGCGATGCTGACTACCCCTGGCCTCGTCAATGCTGTGTTATGAACAGCCTTAAAGAACCTCTC
176 R  T  E  N  S  D  A  D  Y  P  W  P  R  Q  C  C  V  M  N  S  L  K  E  P  L

662 AACCTGGACGCCTGCAAATTAGGAGTGCCTGGATACTACCATAGTCATGGCTGCTATGAGCTGATCTCTGGACCA
201 N  L  D  A  C  K  L  G  V  P  G  Y  Y  H  S  H  G  C  Y  E  L  I  S  G  P

737 ATGAACCGACATGCCTGGGGAGTTGCATGGTTTGGATTTGCCATTCTCTGTTGGACTTTCTGGGTTCTCCTGGGT
226 M  N  R  H  A  W  G  V  A  W  F  G  F  A  I  L  C  W  T  F  W  V  L  L  G

812 ACCATGTTCTACTGGAGCAGAATTGACTATTAAGAATGAAGTGTATGCACCATACCACTCCCCACAGTGACTTTG
251 T  M  F  Y  W  S  R  I  D  Y  *

887 GATTTGGTGCTGGAAATGCTGTCTCCTAATGTTCTACCTTTGTGCTGCCCGGGAACTTACGCATTCTTCCTACAT
962 TGCCAAGTACGTTGGTATGGGGTTCCTTTAAGCTCTCAGACTCTGAAATTTTCAGCACATGTGTTTTCACCCTGA
1037 TCTAGGATTCTGCAACATTGTTATAGACTGTAGGAAAGGGAGGATTTAGGATAGTAGATAATAACTATTCCCATC
1112 TTTGTTTATTTTAATGTGGGGGCATAAAGACAATTCCTAGGAACCTGTGTTATACTGCAAGCAAGTACTGTCATC
1187 GGACAGCAAATCTGCCTGTATTTCTCACTGCTTTCTAAAAGTACCCTG...
1262 ...
1337 ...
1412 ...
1487 CAGCAGCAGCAGCAAAGGCTTTCATTGTATCAGTATTGTCCCAGTGAGAGAACTAAGGAGAAGACTGCTGAAACA
1562 TCTTTTGAATTTGTTCTATGGTGGCTCCCCACCTCAAGTGATTCTCTTAAAGCTAGCTTTCACCCTGA
1637 TATTATCCAAGACAAGGCCTGATCTTGAACAAACAGTGGTTGAAATTTCCTCTCAGACACTGCAGAGTAATTCAT
1712 GCTGGTAACCTCAATTCTCCCACTAATTAAAAGTACGTGAACTTTTGGGACAAAGGAGAGACCTGTTACACATTT
1787 ACCACCTTCAACCTAAAACTGCTTTCCAACAGGGAAGAAGCAAGCCAGCTGTTACTTAGGTGATTTAGGGTGATC
1862 TGTGCACTGCAAAATATTTTTCTTCTGACTCTGTTTCCTTTTGTGATCCTGAAGGAATTTCTTATAACAACATTTG
1937 TCTTTATATATAAATAAAGAGAGTTTTAAAT(A)n
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Figure 5. The deoxynucleotide and deduced amino acid sequence of bovine uroplakin Ib (UPIb). The deduced NH$_2$-terminal amino acid sequence (underlined) matches one of the two NH$_2$-terminal sequences of 27- to 28-kD UPI. The N-linked glycosylation site is circled, and the putative polyadenylation signal AATAAA is underlined by a thick bar. The dash-underlined 3'-untranslated region represents a bovine Alu-like repetitive sequence. Other symbols are as in Fig. 4.

quence is shown in Fig. 5. This 1,965-bp cDNA sequence contains a 61-bp 5'-untranslated sequence, a single open reading frame encoding a protein of 260 amino acids, followed by a relatively long 3'-untranslated region of 1,124-bp containing a bovine Alu-like repetitive sequence. The 3'-untranslated region contains a polyadenylation signal with a poly (A) tail, thus establishing the intactness of the 3' end. Interestingly, this cDNA-encoded protein can account for one of the two N terminal sequences, i.e., AKDDSTVR?-FQGLLIFGNVI (compare Fig. 5 and sequence #5 of Fig. 2 *A*). Alignment of the amino acid sequence of the two uroplakins showed a 39% identity in their amino acid sequences (Fig. 6 *a*), even though their noncoding regions are divergent. Hydropathy plots revealed that both proteins possess four stretches of hydrophobic amino acids that are long enough to serve as TMD (Fig. 6, *a* and *b*). The location of these four TMDs in the two uroplakin I molecules is almost identical, in that the first and the fourth TMDs are located near the NH$_2$ and COOH terminus, respectively. TMD 1 and 2 are connected by a relatively short (∼20 amino acids), while TMDs 3 and 4 are connected by a long (∼120 amino acids) hydrophilic loop. The longer loop of each uroplakin I polypeptide contains a potential N-glycosylation site as well as six highly conserved cysteine residues. In contrast, TMDs 2 and 3 are interrupted by an extremely short but

a.

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UPIa: 1 MASAAAATTEKGSPVVVGLLVMGNIILLSGLALFAETVWVTADQYRIYPLMGVSGKDDVFAGAWIAIF 69
UPIb: 1 MA.....KDDSTVRCFQGLLIFGNVIIGMCSIALMAECIFFVSDQNSLYPLLEATNNDDIYAAAWIGMS 64

     70 CGFSFFVVASFGVGAALCRRRSMILTYLILMLIIYIFECASCITSYTHRDYMVSNPSLITKQMLTFYSA 138
     65 VGICLFCSLVLGIVGIMKSNRKILLVYFILMFIVYAFEVASCITAATQRDFFTPNLFLKQMLERYQN 131

    139 DSNQGRE.......LTRLWDRIMIEQECCGTSGPMDWVNFTSAFRATTPEVVFPWPPLCCRRTGNFIPV 200
    132 NSPPNNDDQWKNNGVTKTWDRLMLQDNCCGVKWPVRLAKYTSAFRTENSDADYPWPRQCCVMNSLKEPL 200

    201 NEEGCRLGHLDYLFTKGCFEHIGHAIDSYTWGISWFGFAILMWTLPVMLIAHYFYTTL 258
    201 NLDACKLGVPGYYHSHGCYELISGPMNRHAWGVAWFGFAILCWTFWVLLGTMFYWSRIDY 260
```

b.

c.

d.

Figure 6. Sequence and structural similarities between bovine uroplakins Ia and Ib. (*a*) Alignment of the two amino acid sequences. Identical amino acid residues shared by the two sequences are boxed, and the conserved cysteine residues are marked by asterisks. Gaps (*dotted*) are introduced to maximize the homology. N-linked glycosylation sites are circled. The four horizontal bars mark the hydrophobic, potential transmembrane domains (see below). (*b*) Hydropathy plot of UPIa and UPIb, done with a moving window of 11 amino acids (Kyte and Doolittle, 1982). (*c*) Homology matrix plot of UPIa and UPIb. Note that maximal homology exists in the four hydrophobic domains and in the middle part of the long hydrophilic region. (*d*) Schematic sequence comparison of UPIa and UPIb. The filled boxes represent the putative membrane-spanning domains. Three positively charged amino acids located between the second and third transmembrane domain are indicated by plus signs. The cysteine residues and N-linked glycosylation sites are denoted by vertical bars and circles, respectively.

Table I. Properties of Bovine Uroplakins Ia, Ib, and Ic

| UP | Partial amino acid sequence | Molecular mass (kD) SDS-PAGE | | | Antibodies | | | | AUM-association in situ | Gene locus on bovine chromosome[¶¶] |
		Native	Core	cDNA	Anti-Ia[∥]	Anti-Ib[∥]	Anti-27/28K[∥]	AE31[§]		
Ia	All 6 internal sequences*	27[∥]	25[¶]	28.89[‡‡]	+	−	−	−	?	18
Ib	NH₂ terminus 1‡	28[∥]	26[¶]	29.68[§§]	−	+	+	−	+[∥∥]	1
Ic	NH₂ terminus 2‡	27[§]	19**	?	−	−	−	+	+[§]	?

* Sequences #1 to #6 of Fig. 2 b.
‡ Sequence (5) of Fig. 2 a.
§ Yu et al. (1990).
∥ Based on immunoblotting using monospecific antisera to synthetic peptides (Fig. 8).
¶ Immunoblotting after deglycosylation (Fig. 7, lane 3; Fig. 8 b).
** Lin et al. (unpublished).
‡‡ cDNA data (Fig. 4).
§§ cDNA data (Fig. 5).
∥∥ An affinity-purified polyclonal rabbit antibody to the 27–28 kDa total UPI proteins (Wu et al., 1990).
¶¶ Ryan et al. (1993).

highly charged linker region (Fig. 6 b). The sequence homology and similar spatial arrangement of the TMDs of the two proteins indicate that they are closely related (Fig. 6, c and d). We therefore named them uroplakins Ia (27 kD; Fig. 4) and Ib (28 kD; Fig. 5). The properties of uroplakin I molecules are summarized in Table I (also see Discussion).

Determination of the Transmembrane Topology of Uroplakin Ia by In Vitro Microsomal Insertion Assays

To determine the transmembrane topology of UPIa, we studied its protease accessibility after it had been inserted in vitro into dog pancreatic microsomes (Fig. 7). In this experiment, UPIa cDNA was transcribed and translated in vitro yielding a primary product which migrated as a 25-kD band on SDS-PAGE (Fig. 7, lane 2). Upon co-translational insertion into dog pancreatic microsomes, this protein became larger (∼27 kD; lane 4) which, however, could be converted back to its original size of 25 kD by deglycosylation using endoglycosidase H (Fig. 7, lane 5). This proved that the extra 2-kD equivalents of mass was due to the addition of an N-linked high-mannose oligosaccharide to a luminal domain of UPIa. Digestion of this membrane-inserted UPIa glycoprotein with a mixture of trypsin and chymotrypsin yielded a 26-kD glycoprotein (Fig. 7, lane 6) containing a core protein of ∼24 kD (lane 7). In contrast, extensive digestion of the inserted UPIa molecule was observed if the microsomal vesicles were detergent-treated before the addition of proteases (Fig. 7, lane 8), indicating that membrane integrity is required for such a protection. These results strongly suggest that the two hydrophilic loops face the microsomal lumen (exoplasmic) space thereby protected from extraneously added proteases. According to this model, the NH₂ and COOH termini and the short linker interconnecting TMD2 and TMD3 (Fig. 6) face the cytoplasmic side and therefore should be susceptible to proteolytic digestion. The fact that a 26-kD glycoprotein was generated (Fig. 7, lane 6) suggests that although the NH₂ and/or COOH termini (totalling 1 to 2 kD) were digested, the extremely short linker region (∼5–amino acid residues) was still intact probably due to steric hindrance (see below).

Uroplakin Ia and Ib Are Distinct Glycoproteins with High Mannose Type of Oligosaccharides

The data presented so far suggest the existence of at least two uroplakin I polypeptides. To identify these two molecules at the protein level, we raised rabbit antisera against a synthetic peptide of 14 amino acids corresponding to a sequence located in the middle of the major luminal loop of UPIa (DSNQGRELTRLWDR; residues 139 to 152; see Fig. 6 a), and another peptide of 18 amino acids corresponding to the
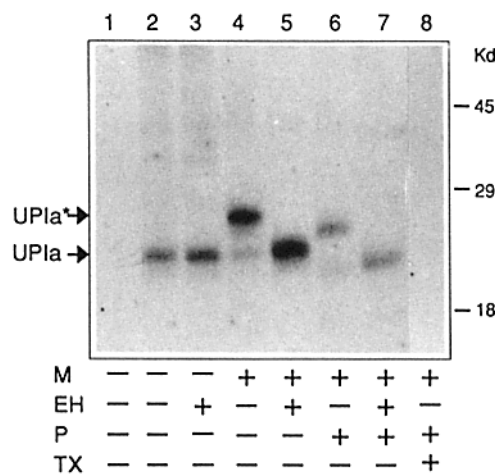

Figure 7. Proteolytic digestion of uroplakin Ia inserted in vitro into microsomal vesicles. In vitro transcribed uroplakin Ia mRNA was translated in the absence (−; lanes 1 to 3) or presence (+; lanes 4 to 8) of dog pancreative microsomes (M). Lane 1 shows a control with no mRNA. After the translation was completed, some samples were dissolved in 1% SDS and treated with endoglycosidase H (EH; lanes 3, 5, 7); some were digested with a mixture of trypsin and chymotrypsin (P; lanes 6 to 8) or protease plus endoglycosidase H (lane 7). Microsomal vesicles of lane 8 were solubilized in 0.5% Triton X-100 before the proteases were added. Note the formation of nascent (core) 25-kD UPIa protein (lane 2), its conversion to a 27-kD glycosylated form of UPIa* (lane 4), the effect of deglycosylation (lane 5), and the partial digestion of UPIa* by proteases resulting in the formation of a 26-kD product (lane 6) with a 24-kD core protein (lane 7).

NH₂-terminal sequence of UPIb (AKDDSTVRCFQGLLI-FGN; residues to 2 to 19; Fig. 6 a). These two peptides were synthesized because according to cDNA sequence data they should be unique to the two UPIs (Fig. 6 a). We found that antisera against these two peptides were indeed monospecific recognizing separately, in total AUM proteins, a 27-kD UPIa and a 28-kD UPIb, which are only partially resolved by SDS-PAGE (Fig. 8 a; Table I). Prior treatment of total AUM proteins with endoglycosidase H reduced these two proteins to 25 and 26 kD (Fig. 8 b), indicating that the single, potential N-glycosylation site located in the middle of the luminal loop of the two uroplakin I molecules is most likely glycosylated with the high mannose type of oligosaccharides.

An interesting difference between UPIa and UPIb, as seen in the above deglycosylation experiment, is that only the former formed a homodimer. This dimer is 54 kD; it is recognized by antibodies to UPIa but not by antibodies to UPIb, UPII, or UPIII (Fig. 8 b and data not shown); and it can be destroyed by reduction. No disulfide-stabilized UPIb homodimer was observed, however, under comparable experimental conditions (Fig. 8 b).

The Expression of Both Uroplakin Ia and Ib Is Urothelium Specific

We have shown earlier using antisera to several uroplakins that these proteins, which are the major protein subunits of AUM, are differentiation products of mammalian bladder
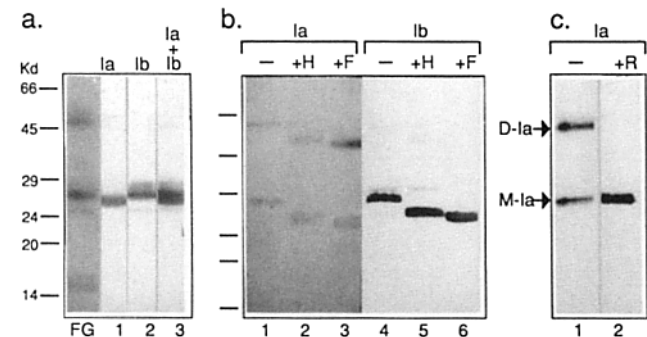
Figure 8. Identification of uroplakins Ia and Ib in purified bovine AUMs by immunoblotting using antibodies raised against synthetic oligopeptides. (a) Proteins of gradient-purified bovine asymmetric unit membrane (AUM) were resolved by SDS-PAGE under reducing conditions, electrophoretically transferred onto a nitrocellulose sheet, and stained with Fast Green (FG). Note the resolution of the 27- to 28-kD UPI, the 15-kD UPII, and the 47-kD UPIII. Similar sheets were stained immunochemically with an antiserum against a UPIa peptide (lane 1), an antiserum against a UPIb peptide (lane 2), or a combination of these two antisera (lane 3). Note the slightly slower electrophoretic mobility of UPIb than UPIa. (b) Immunoblotting of bovine uroplakins treated with buffer alone (lanes 1 and 4) or with endoglycosidase H (+H; lanes 2 and 5) or N-glycosidase F (+F; lanes 3 and 6) using anti-UPIa antiserum (lanes 1 to 3) or anti-UPIb antiserum (lanes 4 to 6). Note that under this nonreducing condition a significant amount of the UPIa (but not UPIb) exists as a disulfide-crosslinked homodimer (see text), and that the electrophoretic mobilities of both UPIa and UPIb increased as a result of deglycosylation. (c) Immunoblotting of non-reduced (lane 1) versus reduced (+R; lane 2) uroplakin Ia. M and D denote monomer and dimer of UPIa, respectively.

epithelium (Surya et al., 1990; Wu et al., 1990; Yu et al., 1990). To determine whether this is true for both UPIa and UPIb and to decide whether the tissue-specificity is reflected at the mRNA level, we probed mRNAs of various bovine tissues with appropriate cDNAs. Abundant UPIa and Ib mRNAs can be detected in bovine bladder epithelium and in cultured bovine urothelial cells (Fig. 9). They are undetectable, however, in esophageal epithelium, snout epithelium, intestinal epithelium, liver, kidney (excluding renal pelvis), lung, and brain (Fig. 9), thus establishing the bladder specificity of UPIa and Ib messages.

Discussion

In this paper, we have established that the 27-kD component of bovine AUM, previously thought to consist of only one protein, actually contains at least two closely related molecules, the uroplakins Ia (27 kD; Fig. 4) and Ib (28 kD; Figs. 5 and 6; Table I). We have also obtained information regarding the transmembrane topology (Figs. 7 and 11), the intra- and intermolecular disulfide cross-linking (Figs. 2, 4, and 8), and the tissue-specific distribution of UPI messages (Fig. 9). Finally, our data indicate that uroplakins Ia and Ib, two major differentiation products of mammalian bladder epithelium, belong to a novel family of membrane proteins all having four membrane-spanning domains (Fig. 10).

Heterogeneity of Uroplakin I

The cDNA-deduced molecular mass of UPIa core polypeptide is 28,882 D (Fig. 4) which, together with ~2 kD corresponding to the high mannose-type oligosaccharide moieties (Fig. 8 b), yields a theoretical molecular mass of ~31 kD. However, UPIa migrates as a 27-kD band on SDS–polyacrylamide gels (Fig. 8 a). Such an under-estimation of the molecular mass by SDS-PAGE has been noted before in other proteins that contain multiple transmembrane domains, e.g., the gap junction proteins (Paul, 1986; Zhang and Nicholson,
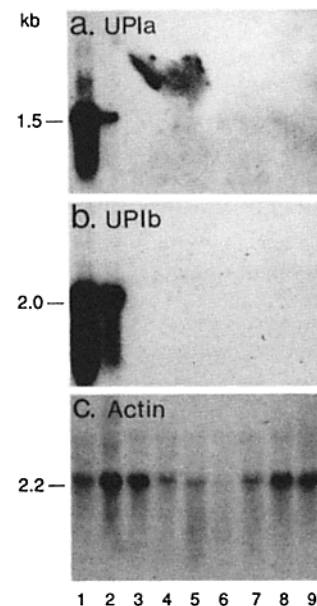
Figure 9. Urothelium-specific distribution of UPIa and UPIb messenger RNAs. a to c show Northern blot analyses of RNAs from different tissues and cultured cells, all of bovine origin unless noted otherwise. Total RNAs (20 μg) from bladder epithelium (lane 1), cultured urothelial cells (lane 2), esophageal epithelium (lane 3), snout epithelium (lane 4), intestinal epithelium (lane 5), liver (lane 6), kidney (lane 7), lung (lane 8), and brain (lane 9) were fractionated on a 1% agarose gel containing formaldehyde, transferred onto a nylon filter, and hybridized with UPIa (a), UPIb (b) or human β-actin cDNA (c).
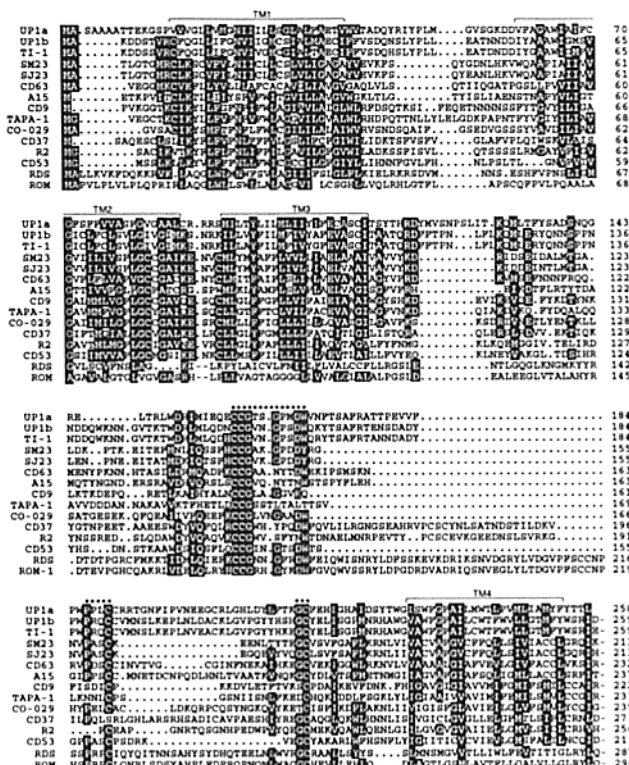
Figure 10. Protein sequence alignment of bovine UPIa and UPIb with members of a family of proteins all containing 4TM domains. The amino acid sequences of 15 related proteins are aligned by the method of Pearson and Lipman (1988). Dots indicate gaps introduced to maximize the alignment. The amino acids that are identical or substitution conserved in at least nine proteins are highlighted in black boxes. Amino acids in one of the following parentheses are considered as substitution conserved: (*E* and *D*), (*K*, *R*, and *H*), (*I*, *L*, *M*, and *V*), (*F*, *W*, and *Y*), (*S* and *T*), and (*A* and *G*). Four potential transmembrane domains are marked TM1 to TM4, which are defined according to the hydropathy plot of UPIa (Figs. 4 and 6). The three highly conserved cysteine-containing motifs are marked by asterisks. The proteins presented here are (the total number of amino acid residues present in each polypeptide is shown in parentheses): *TI-1* (260 amino acids; Kallin et al., 1992); *SM23* and *SJ23* (218 amino acids; Wright et al., 1990; Davern et al., 1991); *CD63* or *ME491* (238 amino acids; Hotta et al., 1988; Metzelaar et al., 1991); *A15* (244 amino acids; Emi et al., 1993); *CD9* (228 amino acids; Lanza et al., 1991); *TAPA-1* (236 amino acids; Oren et al., 1990); *CO-029* (237 amino acids; Szala et al., 1990); *CD37* (281 amino acids; Classon et al., 1990); *R2* (267 amino acids; Gaugitsch et al., 1991); *CD53* (219 amino acids; Amiot, 1990); *RDS* or peripherin (346 amino acids; Travis et al., 1991); and *ROM-1* (351 amino acids; Bascom et al., 1992). The hydrophilic loops interconnecting the TMD2/TMD3 of RDS and ROM-1 (denoted by "--") are much longer than those of other proteins. Although it may not be apparent here, the sequences of UPIa and UPIb/TI-1 are much more similar to each other than to other members of the family.

1990). This protein is recognized specifically by an antiserum against a peptide synthesized according to the cDNA-deduced amino acid sequence of UPIa (Fig. 8 *a*, lane *I*). The fact that this antiserum reacts specifically with UPIa confirms the validity of the corresponding cDNA sequence, and proves that UPIa and UPIb are immunologically distinct (Fig. 8 *a*). Since this anti–UPIa antiserum does not work

well for EM localization, we do not yet have formal proof that UPIa is AUM associated in situ. However, given the facts that UPIa is present in significant amounts in highly purified AUMs (Fig. 8 *a*) and that the closely related UPIb has been shown to be AUM associated (see below), there is little doubt that UPIa is a major subunit of AUM. We have recently demonstrated that the antisera against bovine UPIa can crossreact with a similar 25-to 27-kD AUM protein of a number of mammalian species, suggesting that this protein is highly conserved during mammalian evolution (Wu et al., 1994).

The cDNA-deduced amino acid sequence of uroplakin Ib can account for one of the two NH$_2$-terminal sequences, i.e., '(M)AKDDSTVRCFQGLLIFGNVI', of the 27- to 28-kD AUM component (compare the NH$_2$-terminal sequence of UPIb shown in Fig. 5 with sequence #5 of Fig. 2 *A*). Since none of the members of the 4TM superfamily, to which UPIa and UPIb belong (see below), is known to have a pre-sequence, this result strongly suggests that methionine of the above sequence is the translation–initiation site. The calculated molecular mass of UPIb according to the cDNA sequence is 29,677 D (Fig. 5) which, together with ∼2 kD corresponding to the high mannose-type oligosaccharides (Fig. 8 *b*), yields a theoretical mass of ∼32 kD. This is again significantly higher than the SDS-PAGE–determined value of 28 kD (Fig. 8 *a*). An antiserum raised against a synthetic peptide corresponding to the NH$_2$ terminus of UPIb reacts specifically with the 28-kD UPIb without cross-reacting with the 27 kD UPIa (Fig. 8 *a*), thus confirming that these two uroplakins are distinct. In addition, we have shown recently that an affinity-purified polyclonal antiserum, which was originally raised against total 27- to 28-kD UPI polypeptides and used to prove the AUM association of "UPI" epitopes (Wu et al., 1990), is monospecific for UPIb (Wu et al., 1994). This explains why the screening of an expression library using this polyclonal antiserum yielded a UPIb clone (Fig. 5). With the above and several other antisera prepared against synthetic peptides, we have recently shown that UPIb is present in AUMs of nine different mammalian species indicating that UPIb, like UPIa, is conserved during mammalian evolution (Wu, X.-R., manuscript submitted for publication).

While UPIa and UPIb can account for many important features of the 27- to 28-kD AUM component, they cannot account for the second NH$_2$-terminal sequence of the 27- to 28-kD component (sequence #5 of Fig. 2 *A*). By subtracting the NH$_2$-terminal sequence of UPIb from the NH$_2$-terminal sequences of the 27- to 28-kD component, we can deduce a second sequence: DF?I?WLSGLLSPVMTEL?L. Interestingly, we found that this sequence was identical to the predominant NH$_2$-terminal sequence of the 27 kD, AE31 antigen (data not shown). Moreover, we found recently that the AE31 antigen can be deglycosylated by endoglycosidase H giving rise to a 19-kD core protein (Lin, J.-H., and T.-T. Sun, unpublished results), which is much smaller than the 25-kD core protein of UPIa, and the 26-kD core protein of UPIb. Our earlier EM-localization studies showed that this AE31 antigen, like UPIb, is AUM-associated in situ (Yu et al., 1990). Work is in progress to further characterize this third uroplakin I species (uroplakin Ic; see Table I).

The stoichiometry of the above three uroplakin I molecules cannot be assessed accurately at the present time due

to their incomplete resolution during one- and two-dimensional gel electrophoreses (Fig. 8 *a*; and Yu et al., 1990). However, the fact that all six internal amino acid sequences derived from the two major cyanogen bromide and two major tryptic peptides of the 27- to 28-kD protein (Fig. 2) came from UPIa suggests that this is the predominant species. That the NH$_2$-terminal sequence of UPIa was not among the two experimentally determined raises the possibility that UPIa may be N blocked.

UPIa and UPIb Belong to a Family of Four Transmembrane Domain Proteins

A comparison of the amino acid sequences of UPIa and UPIb with those in the GenBank revealed that the UPIb sequence is 93% identical to a hypothetical protein encoded by the TI-1 gene of a mink lung epithelial cell line (CCL64). Kallin et al. (1992) cloned the cDNA of this gene by differential screening of a cDNA library of growth-arrested cells, and showed that the mRNA level of TI-1 is higher in cells growth arrested by serum starvation. The high degree of sequence identity strongly suggests that TI-1 is the mink homologue of bovine uroplakin Ib. That this particular lung epithelial cell line can express uroplakin Ib is intriguing since so far we have not been able to detect UPIb in bovine lung by immunohistochemical staining or by Northern blot analysis (Fig. 9; and unpublished results). More experiments are needed to determine whether CCL64 cells originate from a subpopulation of UPIb/TI-1–positive mink lung epithelial cells. In any case, the fact that TI-1 mRNA level increases in growth-arrested CCL64 cells (Kallin et al., 1992) parallels our observation that in bovine urothelial culture, in which cells form stratified and differentiating colonies, uroplakin synthesis occurs mainly in terminally differentiated cells (Surya et al., 1990; Yu et al., 1990, 1992). This raises the possibility that the uroplakin Ib/TI-1 protein is synthesized as a differentiation product in certain lung epithelial cells.

Computer search of the GenBank also established that UPIa and UPIb/TI-1, which are significantly homologous to each other (Fig. 6), belong to a growing family of integral membrane proteins that possess four transmembrane domains (Fig. 10; also see Kallin et al., 1992, who first noted that TI-1 belongs to this family). Members of this family (reviewed by Horejsi and Vlcek, 1991) contain four potential transmembrane domains, of which three are located in the NH$_2$-terminal portion and the fourth is near the COOH terminus (Figs. 6 and 10). The intron/exon locations of several members of this gene family, including bovine UPIa, are highly conserved (Wright et al., 1993; Lin, J.-H., and T.-T. Sun, manuscript in preparation). The amino acid sequences of the TMDs, particularly the first three, are moderately conserved (Fig. 10). In addition, the major hydrophilic loop interconnecting the third and fourth TMDs of all these proteins share several highly conserved cysteine-containing motifs (Fig. 10). One of them, CCGXXXXXDW, appears to be highly specific for the 4TM family. Although ROM-1 and RDS/peripherin, two retina-specific membrane proteins, also have four TMDs and their second luminal loops also have this particular motif (Travis et al., 1991; Bascom et al., 1992), they do not belong to the 4TM family because the intron/exon locations of their genes (Bascom et al., 1993) are

clearly distinct from the common pattern shared by the genes of the 4TM family (Wright et al., 1993; Lin, J.-H., and T.-T. Sun, in preparation). Another highly conserved motif is PXXC. Although the P of this motif is missing in CD9, TAPA-1 and R2, this appears to be compensated by a proline residue located on the COOH-terminal side of the cysteine (Fig. 10). This suggests that the association of this cysteine with a proline-induced bend in the polypeptide backbone is critically important. It is thus curious as to why CD37 lacks this cysteine altogether. Finally, a GC motif exists toward the COOH-terminal and of the major luminal loop (Fig. 10).

The members of this 4TM family include, in addition to UPIa and UPIb, ME491/CD63, a melanoma- and activated platelet-associated surface antigen (Hotta et al., 1988; Metzelaar et al., 1991); A15, a hypothetical protein whose expression is elevated in activated T cell lines (Emi et al., 1993); CD9, a surface antigen expressed by a variety of hematopoietic and epithelial cells (Boucheix et al., 1991; Lanza et al., 1991); TAPA-1, a cell surface protein present in many human cell lines (Oren et al., 1990); CO-029, a glycoprotein of gastric, colon, and renal carcinoma (Szala et al., 1990); CD37, a glycoprotein abundantly expressed in B lymphocytes (Classon et al., 1990); R2, a membrane protein of activated lymphocytes (Gaugitsch et al., 1991); CD53, a cell surface glycoprotein of panleukocytes (Amiot, 1990); and even SM23 and SJ23, two surface antigens of *Schistosoma* (Wright et al., 1990; Davern et al., 1991). The biological significance of this 4TM family is currently unclear. It has been shown, however, that the expression of some of its members varies depending on cellular growth and differentiation (Gaugitsch et al., 1991; Kallin et al., 1992; Nishikata et al., 1992; Emi et al., 1993), and that monoclonal antibodies to some of these proteins can modulate cell growth (TAPA-1; Oren et al., 1990) or adhesion (CD9; Masellis et al., 1990). In this regard, UPIa and UPIb/TI-1 are unique in that they are exceptionally tissue restricted, being synthesized as the major differentiation products of the urothelium (Fig. 9). Moreover, as a part of the uroplakin protein complex, they can be prepared readily in a naturally occurring, crystalline state in milligram quantities thus providing unique opportunities for detailed structural analyses (Walz, T., X.-R. Wu, T.-T. Sun and U. Aebi, manuscript in preparation). The fact that uroplakin I molecules apparently interact tightly with a smaller molecular weight uroplakin II of 15 kD is of particular interest, in that two other 4TM family members are known to share this feature. Thus CD9 can associate with a 14.5-kD diphtheria toxin receptor resulting in enhanced ligand binding (Mitamura et al., 1992). The TAPA-1 interacts with a 16-kD leu-13 protein to form a complex possibly involved in signal transduction (Takahashi et al., 1990; Bradbury et al., 1992). Whether other members of the 4TM family also interact with a protein of 14 to 15 kD is unknown.

Transmembrane Topology of Uroplakins Ia and Ib

Our data on the proteolytic digestion of UPIa inserted in vitro into dog pancreatic microsomes strongly suggest that the two hydrophilic loops of UPIa face the luminal space (Figs. 7 and 11). The transmembrane topology of the structurally related UPIb is almost certainly the same. This topology is supported by the fact that the single N-glycosylation site located in the major hydrophilic loop of UPIa and UPIb

appears to be glycosylated (Fig. 8 *b*), and by our previous EM localization data showing that an affinity-purified anti-UPIb antiserum labeled exclusively the outer leaflet of apical AUM plaques (Wu et al., 1990). Additional support for this interpretation came from earlier studies on several other members of the 4TM family. Levy et al. (1991) studied the protease susceptibility of TAPA-1 inserted in vitro into microsomes and showed that its two hydrophilic loops are protected. Tomlinson et al. (1993) showed more recently that monoclonal antibodies to the major hydrophilic loop of CD53 can recognize the antigen of living cells. These results are consistent with ours and suggest that the transmembrane topology of all members of the 4TM family is likely to be the same.

We have previously shown that bovine uroplakin II is synthesized as a 28-kD precursor containing a cleavable signal peptide as well as a relatively long and heavily glycosylated pro-sequence (Lin et al., 1994). The mature uroplakin II is 100-amino acid residues long containing a single potential transmembrane domain located at its very COOH-terminus. Proteolytic digestion of in vitro translated/transcribed UPII that has been inserted into dog pancreatic microsomes indicates that the NH_2-terminal bulk of the UPII molecule is located on the luminal side (type I topology; Lin et al., 1994; Fig. 11). Molecular characterization of the 47-kD bovine uroplakin III has established that it is also a type I protein containing a single transmembrane domain and assuming an N_{exo}/C_{cyto} configuration (Wu and Sun, 1993). Moreover, our data indicate that, of all the major AUM components, UPIII is the only one having a significant cytoplasmic domain. This domain contains multiple, potential phosphorylation sites, which can potentially be involved in regulating AUM/cytoskeletal interaction (Fig. 11; Wu and Sun, 1993).

Thus, an important feature shared by all major uroplakins (Fig. 11) is that they all have large exoplasmic domains with very small cytoplasmic ones. This is true for UPIa, UPIb, and UPII (Fig. 11). Even for UPIII, the mass of its exoplasmic domain (17-kD core protein plus 20-kD equivalents of oligosaccharides) greatly exceeds its cytoplasmic one ($\sim$5-kD). Since these four uroplakins (UPIa, UPIb, UPII, and UPIII) can account for >90% of the total proteins of highly purified AUMs exhibiting crystalline patches of 12-nm particles, it seems clear that the extracellular domains of some of these major AUM subunits must form the bulk of such exoplasmic particles. The exceedingly high ratio of exoplasmic vs. cytoplasmic domains of uroplakins provides a possible explanation for the observation that the 12-nm protein particles are associated exclusively with the outer leaflet (Robertson and Vergara, 1980; Brisson and Wade, 1983; Taylor and Robertson, 1984).

The Major Luminal Loop of Uroplakin Ia Is Stabilized by Intramolecular Disulfide Bonds

As mentioned earlier, the major hydrophilic loop of all members of the 4TM proteins contain several highly conserved cysteine residues. Several pieces of evidence suggest that some of these residues are involved in forming intramolecular disulfide bridges. Oligino et al. (1988) showed that an epitope in the major loop of SM23 is destroyed by reduction. Levy et al. (1991) showed that a monoclonal antibody to TAPA-1 fails to recognize in vitro transcribed/translated TAPA-1 unless it is inserted into dog pancreatic microsomes.

ASYMMETRICAL MASS DISTRIBUTION OF UROPLAKINS I, II AND III IN ASYMMETRICAL UNIT MEMBRANE
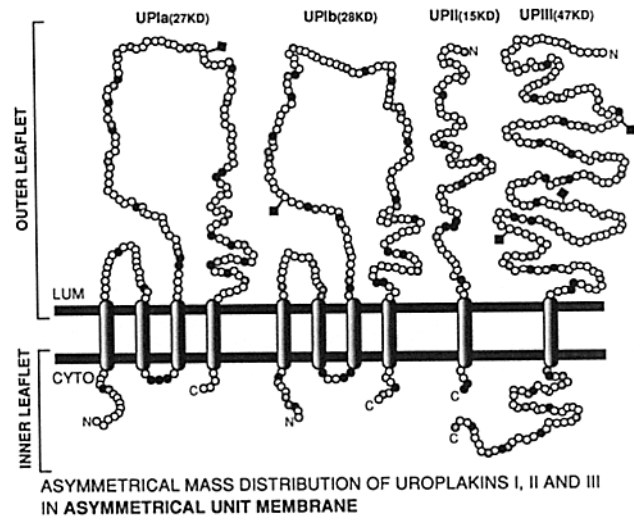
Figure 11. The hypothetical transmembrane topology of Uroplakins Ia, Ib, II, and III. The transmembrane topology of UPIa and UPIb is based on data shown in Figs. 4, 5, 6, and 7, while that of UPII and UPIII is based on Lin et al. (1994) and Wu and Sun (1993), respectively. The potential transmembrane domains (TMD) of uroplakins are represented by thick vertical bars. N and C denote the NH_2 and COOH terminus, respectively, of the proteins. The lipid bilayer is represented by the two horizontal bars, with its luminal (*lum*) and cytoplasmic (*cyto*) leaflets labeled. The positively and negatively charged amino acid residues are represented by filled and gray circles. Filled boxes denote potential N-glycosylation sites. Note that according to this hypothetical scheme UPIa, UPIb, and UPII have practically no cytoplasmic domains, and that even for UPIII its extracellular domain greatly exceeds its cytoplasmic domain (which may be involved in AUM/cytoskeletal interaction; see Wu and Sun, 1993).

In addition, intramolecular disulfide bond(s) is (are) important for maintaining its antigenicity. Tomlinson et al. (1993) also showed that monoclonal antibodies to an epitope of the hydrophilic loop of rat CD53 antigen is sensitive to reduction/alkylation.

One of the two major cyanogen bromide products of the 27- to 28-kD UPI component (C2, see Fig. 1 *a*, lane *4*) contained two NH_2-terminal sequences (Nos. 3 and 1; Figs. 2 *B* and 4) which correspond to a 1.2-kD peptide [IE-QECCGTSGPM] and a $\sim$7.6-kD peptide located immediately downstream [DWVNFTSAF . . . GPAILM]. The co-electrophoresis of these two peptides of greatly different size during SDS-PAGE under a non-reducing condition suggests that they are disulfide cross-linked. While in peptide No. 3 the doublet CC are the only cysteines that can be involved in this cross-linking, more data are needed to determine which of the four cysteines in peptide No. 1 is cross-linked.

Concluding Remarks

Although proteins moderately homologous to UPIa and UPIb are widespread, only urothelium makes the characteristic crystalline arrays of 12-nm protein particles. Since the sequences of the luminal loops of UPIa and Ib are quite distinct from those of the other 4TM family members, these UPI sequences may be involved in interacting specifically with the UPII and UPIII to form the AUM particles. As one of the approaches to study these interactions in detail, we have generated a panel of rabbit and chicken antisera against

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many oligopeptides synthesized according to the cDNA-derived amino acid sequences of not only UPIa and UPIb (Fig. 8), but also uroplakin II (Lin et al., 1994) and uroplakin III (Wu and Sun, 1993). Immunolocalization of these epitopes on purified AUMs coupled with digital image analysis may allow us to assign specific epitopes to individual domains of the 12-nm protein particles.

The function of uroplakins in general, and of uroplakin I isoforms in particular, has not yet been firmly established. However, the fact that UPIa and UPIb are highly conserved during mammalian evolution, both in terms of their size and epitopes (Wu et al., 1994), suggests that they play an important role in normal bladder epithelial physiology—possibly in regulating membrane permeability of superficial umbrella cells or in stabilizing the apical membrane through AUM/cytoskeletal interactions. Experiments are in progress to better understand how UPI molecules interact with other uroplakins to form the 12-nm protein particles, and to eventually understand the functional role of various uroplakin I molecules by gene targeting and other transgenic approaches.

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