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.

The 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.

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 Bonville, 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 Massexo/Massemo. 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 NH2-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 NH2-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)+ mRNA were purified using an oligo-dT cellulose column (type 3; Collaborative Research, Boston, MA). Single-stranded cDNAs of these mRNA were synthesized using avian myeloblastosis 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: FEVFPP, MLFTYS, and DLYFTK 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 Tag 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-7Z (Promega Biotec, Madison, WI) and sequenced using a T7 DNA Sequencing Kit (Version 2; US Biochemical, Cleveland, OH). Specific PCR products were 32P-labeled and used to screen a kgt10 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 denaturated salmon sperm DNA. cDNA inserts from positive plaques were subcloned into pGEM7Z and sequenced. The immunological screening of a gkt11 expression library was done according to standard methods (Sambrook et al., 1989). The cDNA inserts of positive clones were subcloned into pGEMZ 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 32P-labeled UPI or UPIb cDNA at 42°C overnight in 6× SSC, 50% formamide, 1× Denhardt’s solution, 0.1% SDS, 10% dialyzed, 0.1 M EDTA, and 50 μg/ml denatured salmon sperm DNA. The filter was then washed at 65°C in 0.2× SSC and 0.1% SDS for 30 min. In some cases, the probe was stripped off by washing the filter in 50% formamide and 2× SSC at 80°C for 1 h followed by rehybridizing with a 32P-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 32P-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, "DSNGRELTRLWD" and "AKDDSTVSPQGLLPQN", 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 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 key-hole limpet hemocyanin (KLH) or bovine serum albumin using m-maleimidobenzul-N-hydroxysuccinimide (MBS). 100 μg of the conjugated peptides 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 t[35S]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 containing 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. 1a, 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. 1a, lane 3) with trypsin and cNBr 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 J) 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.

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A. Primary Data:

(1) C1
(D/E/L-V/Q/R)-[Y/F]-[V/Y]-[E/F]-[I/V]-[E/F]-[A/K]-[E/F]-[A/Q]-[A/D]-[T/E]-[T/C]
(2) C2
(D/E)-[E/Q]-[Q/E]-[F/R]-[V/R]-[S/D]-[N/S]-[D/A]-[N/R]-[S/D]-[V/R]-[V/R]-[Q/F]-[E/F]
(3) T1
(D/E)-[K/M]-[V/Y]-[E/F]-[T/K]-[N/A]-[N/V]-[F/S]-[Y/M]-[S/P]-[Y/N]-[D/L]-[D/L]-[N/I]-[T/I]
(4) T2
(Q/E)-[K/M]-[V/Y]-[E/F]-[T/K]-[N/A]-[N/V]-[F/S]-[Y/M]-[S/P]-[Y/N]-[D/L]-[D/L]-[N/I]-[T/I]
(5) NH2
(D/E)-[K/M]-[V/Y]-[E/F]-[T/K]-[N/A]-[N/V]-[F/S]-[Y/M]-[S/P]-[Y/N]-[D/L]-[D/L]-[N/I]-[T/I]

B. Deduced Sequences:

#1 C1 and C2
D-(E/Q)-[Y/F]-[V/Y]-[E/F]-[I/V]-[E/F]-[A/K]-[E/F]-[A/Q]-[A/D]-[T/E]-[T/C]
#2 T1 only
L-P-Y-T-R-A-D-W-Q-O-I
#3 T2 only
L-W-E-Y-V-S-A-D-S-N-O-G
#4 T1, T2 and #2
G-D-(E/Q)-[Y/F]-[V/Y]-[E/F]-[I/V]-[E/F]-[A/K]-[E/F]-[A/Q]-[A/D]-[T/E]-[T/C]
#5 T1 only
D-Y-X-V-S-N-P-L-I-T-K
#6 T2 only
L-G-H-L-D-Y-L-A-T-T-P-R-V-V-F-P-

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" sequences, 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 (l to 3), degenerate oligonucleotide primers were synthesized 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/Q)-[Y/F]-[V/Y]-[E/F]-[I/V]-[E/F]-[A/K]-[E/F]-[A/Q]-[A/D]-[T/E]-[T/C] (Fig. 2 B, sequence l), 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 l). 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 J) but not in 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 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 Agt1 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-
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.

Figure 5. The deoxynucleotide and deduced amino acid sequence of bovine uroplakin Ib (UPIb). The deduced NH₂-terminal 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.
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 

|     |                             | Molecular mass (kD) | Native | Core | cDNA | Anti-Ia | Anti-Ib | Anti-27/28KH | AE31 | ? | + | - | - | - | - | \+ | \+ | - | - | - | - | 18 |
| Ia  | All 6 internal sequences*   | 271                 | 251    |     | 28.89** | +     | -     | -     | -     | -     | ?    | + | - | - | - | - | - | - | - | - | - | 18 |
| Ib  | NH₂ terminus†               | 281                 | 261    |     | 29.68| -     | +     | -     | -     | -     | -     | + | + | - | - | - | - | - | - | - | - | 1 |
| Ic  | NI-I2 terminus‡             | 271                 | 19**   | ?    | -     | -     | -     | -     | -     | +     | +     | - | - | - | - | - | - | - | - | - | - | 18 |

* 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).
¶¶ Ryan et al. (1993).
H An affinity-purified polyclonal rabbit antibody to the 27-28 kDa total UPI proteins (Wu et al., 1990).

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 pancreatic microsomes (M). Lane I 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).]
proteins with endoglycosidase H reduced these two proteins and a 28-kD u-Pro, which are only partially resolved by recognizing separately, in total AUM proteins, a 27-kD UPIa synthesized because according to cDNA sequence data they NH2-terminal sequence of UPIb (AKDDSTVRCFQGLLIFGN; residues to 2 to 19; Fig. 6 a). These two peptides were indeed monospecific recognizing separately, in total AUM proteins, a 27-kD UPIa and a 28-kD UPb, 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 UPb, 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 UPb, UPII, or UPIII (Fig. 8 b and data not shown); and it can be destroyed by reduction. No disulfide-stabilized UPb 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 epithelium (Surya et al., 1990; Wu et al., 1990; Yu et al., 1990). To determine whether this is true for both UPIa and UPb 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 intramembrane 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 ψ2 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, 1986). Urothelium-specific distribution of UPIa and UPb 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), UPb (b) or human β-actin cDNA (c).
Proves that UPIa and UPIb are immunologically distinct. The cDNA-deduced amino acid sequence of UPIa (Fig. 8 a, lane D) 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, 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 NH₂-terminal sequences, i.e., "(M)AKDDSTVRCFQGLLIFGNVT", of the 27- to 28-kD AUM component (compare the NH₂-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 ~25 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 NH₂ 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 uropakins 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 nonspecific 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 NH₂-terminal sequence of the 27- to 28-kD component (sequence #5 of Fig. 2 A). By subtracting the NH₂-terminal sequence of UPIb from the NH₂-terminal sequences of the 27- to 28-kD component, we can deduce a second sequence: DB?IDSLPSVMTL7L. Interestingly, we found that this sequence was identical to the predominant NH₂-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-dimen-
sional 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 tryptic peptides of the 27- to 28-kD protein (Fig. 2) came
from UPIa suggests that this is the predominant species. That
the NH2-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 im-
munohistochemical 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) paral-
lels our observation that in bovine urothelial culture, in
which cells form stratified and differentiating colonies, uro-
plakin 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 synthe-
sized 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 do-
 mains (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
NH2-terminal portion and the fourth is near the COOH ter-
minus (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 con-
served (Fig. 10). In addition, the major hydrophilic loop in-
terconnecting the third and fourth TMDs of all these proteins
share several highly conserved cysteine-containing motifs
(Fig. 10). One of them, CCGXXXXXXDW, 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 in-
tron/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
PXCC. 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; Met-
zelaa et al., 1991); A15, a hypothetical protein whose expres-
sion 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 gly-
coprotein of gastric, colon, and renal carcinoma (Szala et al.,
1990); CD37, a glycoprotein abundantly expressed in B lym-
phocytes (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 differenti-
ation (Gaugitsch et al., 1991; Kallin et al., 1992; Nishikata
et al., 1992; Emi et al., 1993), and that monoclonal antibod-
ies 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 synthe-
sized as the major differentiation products of the urothelium
(Fig. 9). Moreover, as a part of the uroplakin protein com-
plex, 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 prepara-
tion). 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 mem-
bers are known to share this feature. Thus CD9 can associate
with a 14.5-kD diphertheria toxin receptor resulting in en-
hanced ligand binding (Mitamura et al., 1992). The TAPA-1
interacts with a 16-kD leu-13 protein to form a complex pos-
sibly 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 struc-
turally related UPIb is almost certainly the same. This topol-
ogy is supported by the fact that the single N-glycosylation
site located in the major hydrophilic loop of UPIa and UPIb

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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 NH2-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\textsubscript{exo}/C\textsubscript{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 (~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.

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\textsubscript{2}-terminal sequences (Nos. 3 and 1; Figs. 2 B and 4) which correspond to a 1.2-kD peptide [IE-QECCTGSPM] and a ~7.6-kD peptide located immediately downstream [DWVNTSAP ... 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
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 uropolakins 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 uropolakins 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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