Uroplakin I: A 27-kD Protein Associated with the Asymmetric Unit Membrane of Mammalian Urothelium

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Abstract. The luminal surface of mammalian urothelium is covered with numerous plaques (also known as the asymmetric unit membrane or AUM) composed of semi-crystalline, hexagonal arrays of 12-nm protein particles. Despite the presumed importance of these plaques in stabilizing the urothelial surface during bladder distention, relatively little is known about their protein composition. Using a mouse mAb, AE31, we have identified a 27-kD protein that is urothelium-specific and is differentially expressed in superficial umbrella cells. This protein (pI ∼5.8) partitions into the detergent phase during Triton X-114 phase separation. Pulse–chase experiments using cultured bovine urothelial cells showed that this protein is synthesized as a 32-kD precursor that is processed through a 30-kD intermediate, to the mature 27-kD form. In cytoplasmic vesicles containing immature AUM, the AE31 epitope is detected in patches on the cytoplasmic side, but in mature, apical AUM it is detected exclusively on the luminal side. This suggests an unusual translocation of the AE31 epitope during AUM maturation; more data are required, however, to substantiate this interpretation. Immunoaffinity purification of the 27-kD protein results in the copurification in approximately molar ratio of a 15-kD protein, as well as a small and variable amount of a 47-kD protein. Immunoblotting data indicate that these three proteins are immunologically distinguishable. This copurified 15-kD protein is relative basic (pI ∼8.0). Like the 27-kD protein, it is urothelium-specific and is present mainly in the umbrella cells. Together, our data indicate that a 27-kD protein is *urothelial plaque*-associated (uroplakin I). Based on complex formation data, we provisionally name the 15-kD protein uroplakin II; additional data will be required to determine whether this and the 47-kD protein are integral parts of AUM. The identification of these AUM-associated and -related proteins, plus the availability of a culture system capable of synthesizing and processing some of these molecules, offer new opportunities for studying the detailed structure, assembly, and function of asymmetrical unit membrane.

THE luminal surface of mammalian urinary bladder epithelium (also called urothelium or "transitional" epithelium) contains numerous specialized, scallop-shaped plaques that are surrounded and interconnected by a smooth, flexible membrane (the "hinge" area; Porter and Bonneville, 1963). The membrane structure of these plaques (0.1–0.5 μm in diameter) is highly unusual in that its luminal leaflet is almost twice as thick as the cytoplasmic leaflet (8 vs. 4 nm), hence the name "asymmetric unit membrane" (AUM;[1] Hicks, 1965; Koss, 1969). Negative staining and freeze-fracturing of the plaque region reveals numerous particles (12 nm in diameter) arranged in a semicrystalline, hexagonal array (Hicks and Ketterer, 1969, 1970; Vergara et al., 1969; Staehelin et al., 1972; Knutton and Robertson, 1976; Severs and Hicks, 1979). These particles are trypsin-sensitive and are therefore at least partially protein in nature (Caruthers and Bonneville, 1980). They are tightly packed and protrude 5–6 nm into the luminal space thus accounting for the thickened appearance of the outer membrane leaflet (Chlapowski et al., 1972; Staehelin et al., 1972; Robertson and Vergara, 1980; Brisson and Wade, 1983). High-resolution EM, coupled with image analysis, has shown that each particle can be divided into six dumbbell-shaped subdomains that form a hexagonal structure surrounding a central depression (Warren and Hicks, 1970; Robertson, 1972).

The function of these plaque-associated particles remains unclear. It has been suggested, however, that they play a role in establishing a permeability barrier (Hicks, 1966a). Alternatively, these plaques may serve as anchors for an underlying cytoskeletal network; it is presumed that such an interaction can strengthen the membranes thereby preventing them from rupturing during bladder distention (Staehelin et al., 1972; Minsky and Chlapowski, 1978; Sarikas and Chlapowski, 1986). Because these plaques are also present cytoplasmically in numerous discoidal or fusiform vesicles of superficial cells, such cytoplasmic plaques are thought to serve as a reservoir of membrane to be inserted into the lu-

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1. *Abbreviation used in this paper*: AUM, asymmetric unit membrane.

minal surface during bladder distention (Porter and Bonneville, 1963; Hicks, 1966b; Porter et al., 1967; Minsky and Chlapowski, 1978; Lewis and Moura, 1984; however, for the view that many of them represent immature, intermediate stages of plaque maturation, see Severs and Hicks, 1979).

The protein components of the AUM have been analyzed by several groups, with somewhat conflicting results. Using a partially purified rat AUM preparation, Ketterer et al. (1973) found a large number of proteins ranging from 10 to >200 kD. Vergara et al. (1974) purified pig AUM by zonal centrifugation, and detected mainly a broad protein band in the 25–27-kD region by SDS-PAGE analysis. Caruthers and Bonneville (1977) identified a major 33-kD protein in a preparation of detergent-washed, sheep AUM. In the AUM preparations of both Vergara et al. (1974) and Caruthers and Bonneville (1977), a number of minor proteins were also present; however, those in the 40–60-kD regions were thought to represent dimers of the 25–27-kD proteins, while some lower molecular weight proteins were thought to be contaminants and/or degradative products. More recently, Stubbs et al. (1979) identified at least five proteins including a 12- and a 22-kD protein in a bovine AUM preparation. They reported that these two proteins can form a 30-kD complex, which they thought may correspond to the 33-kD component described by Caruthers and Bonneville (1977). Therefore, multiple proteins have been identified in various AUM preparations. However, whether any of these proteins are actually AUM-associated in situ has never been established, and the relationships among different candidate proteins remain obscure.

To identify the protein subunits of asymmetric unit membrane so that we will ultimately be able to utilize these proteins as markers for studying membrane–cytoskeletal interactions and urothelial differentiation, we have generated an mAb to the apical surface of bovine urothelium. Using this antibody, AE31, we have identified a 27-kD urothelium-specific protein and have localized it to the luminal leaflet of apical plaques. Localization of this epitope on AUM-containing, cytoplasmic vesicles indicates that the AE31 epitope undergoes an unusual translocation from the cytoplasmic side of the membrane to the luminal side during AUM maturation. In addition, we provide evidence that this 27-kD protein copurifies with another major 15-kD protein (in approximately equimolar ratio) and a minor 47-kD protein, most likely through complex formation. The identification of these three plaque-related molecules opens new avenues for studying the detailed structure and function of the asymmetric unit membrane.

Materials and Methods

mAb Production

A crude plasma membrane fraction was isolated from bovine bladder epithelium using Percoll gradient (Armstrong and Newman, 1985) and used to immunize Balb/c mice. Hybridoma cells were produced by fusing spleen cells with PAI myeloma cells (Stocker et al., 1982) according to Kohler and Milstein (1975). Hybridoma cells secreting antibodies reacting selectively with bovine urothelial umbrella cells without crossreacting with other tissues were screened by immunofluorescent staining of various frozen tissue sections. Antibodies reacting with exposed, cell surface antigens were screened by staining nonpermeabilized, differentiating cultures of bovine urothelial cells (see below).

Immunofluorescent Staining

Tissues were fixed with Zamboni's fixative (15% "saturated" picric acid and 2% paraformaldehyde in PBS), cut into 6 μm frozen sections, and stained by indirect immunofluorescence (Sun and Green, 1978; O'Guin et al., 1986). Cultured urothelial and other cells were fixed and permeabilized with ice-cold methanol-acetone (1:1) for 5 min and stained similarly. For the selective staining of cell surface, cultured cells were fixed at room temperature for 5 min with 3.7% paraformaldehyde in PBS, washed with PBS, and stained by standard procedures.

Cell Culture

Bovine urothelial cells were scraped from cow bladders, and were grown in a 1:1 mixture of DME and Ham's F12 medium (Gibco Laboratories, Grand Island, NY) supplemented with 20% FCS, 5 μg/ml hydrocortisone, in the presence of mitomycin-treated 3T3 feeder cells (Sun et al., 1980; Wu et al., 1982). Similar urothelial growth and differentiation were obtained in a serum-free medium containing hydrocortisone (5 μg/ml), choleratoxin (5 μg/ml), lamda-aldosterone (1 μg/ml), dihydrotestosterone (1.5 μg/ml), sodium selenite (17.2 pg/ml), testosterone (250 ng/ml), beta-estradiol (300 ng/ml), somatostatin (100 ng/ml) and 3,3,5-triiodo-2-thyroxine (20 pg/ml). Bovine dorsal tongue and esophageal epithelial cells were isolated by treating tissue fragments with dispase (2.5 mg/ml) at 4°C overnight, and then incubating the detached epithelial sheets in 0.1% trypsin, 0.01% EDTA in PBS at 37°C for 30 min. The released, single cells were cultured in DME containing 20% FCS and 5 μg/ml hydrocortisone with mitomycin-treated 3T3 feeder cells (Rheinwald and Green, 1975; Doran et al., 1980). Bovine bladder fibroblasts were grown in DME with 10% calf serum.

Immunoelectron Microscopy

Tissues were fixed with Zamboni's fixative, cut into 4-μm frozen sections, incubated at 37°C for 10 min with 10% normal goat serum in PBS, and reacted with primary antibodies at 37°C for 2 to 4 h (or at 4°C overnight). After washing in PBS for 1 h, the sections were treated at 4°C overnight with a secondary antibody conjugated with 5 nm colloidal gold particles, fixed again with 2.5% glutaraldehyde for 1 h, followed by staining with 1% OsO₄ for 30 min at room temperature. The specimens were then dehydrated in ethanol and acetone, embedded in Epon, sectioned and viewed using an electron microscope.

For conventional EM, tissues were fixed for 2 h each with 2.5% glutaraldehyde and with 1% OsO₄, and then processed routinely.

Triton X-114 Phase Separation

Phase separation with Triton X-114 was carried out according to Bordier (1981), with minor modifications. Briefly, [³⁵S]methionine-labeled, cultured bovine urothelial cells were lysed in Triton X-114 extraction buffer (1% Triton X-114; 50 mM Tris-HCl, pH 7.4; 0.5M NaCl; 1 mM PMSF; 1 mM EDTA; 1 μg/ml each of antipain, pepstatin, and chymostatin), homogenized, and transferred to a 1.5-ml microfuge tube. After the insoluble material was removed by centrifugation (10,000 g, 4°C), the supernatant was again centrifuged at room temperature for 10 min. The Triton X-114 and the aqueous phases were separated. The detergent phase was reextracted with 5 vol of the extraction buffer without Triton X-114, and the aqueous phase was reextracted with one-fifth volume of 15% Triton X-114; both followed by phase separation. The reextraction was repeated three times and the appropriate fractions combined for SDS-PAGE analysis.

Pulse–Chase Labeling of Cultured Bovine Urothelial Cells

Bovine urothelial cells (90% confluent) cultured in serum-free medium were rinsed twice with PBS prewarmed to 37°C and incubated for 30 min in a methionine-free MEM supplemented with hydrocortisone (5 μg/ml), choleratoxin (5 μg/ml), insulin (5 μg/ml), and epidermal growth factor (15 ng/ml). The cells were then pulse-labeled for 10 min by adding 1 mCi [³⁵S]methionine per 10 ml of the same serum-free, methionine-free medium per 100-mm dishes (containing 10⁷ cells). Chasing was done using serum-free medium (see Cell Culture) with an excess of unlabeled methionine (30 μg/ml). The labeled cells were washed with cold PBS, and extracted with lysis buffer (1% Triton X-100, 25 mM Tris-HCl, pH 7.4; 0.5 M NaCl; 1 mM PMSF; 1 mM EDTA; 1 μg/ml of antipain, pepstatin, and chymostatin) for immunoprecipitation.

Immunoprecipitation and Affinity Chromatography

AE31 antibody was purified from a serum-free hybridoma culture supernatant (Nutridoma-NS serum-free medium; Boehringer Mannheim Biochemicals, Indianapolis, IN) by ammonium sulfate precipitation, and was coupled to cyanogen bromide-activated Sepharose 4B according to the manufacturer's recommendations (Pharmacia Fine Chemicals, Piscataway, NJ) with an antibody to swollen beads ratio of 1 mg to 1 ml. Mouse IgG from a secreting myeloma cell line (P3) was also conjugated to serve as a control.

For immunoprecipitation, cultured cells were labeled with [^{35}S]methionine and then lysed with Triton X-100 lysis buffer as described before. After a brief centrifugation, the supernatant was diluted with lysis buffer to 10^6 cpm/ml and incubated, at 4°C overnight with gentle agitation, with 30 μl of a suspension of antibody-conjugated Sepharose 4B beads (50% suspension volume in lysis buffer; preblocked with 3% BSA in lysis buffer). The reaction mixtures were washed five times with lysis buffer omitting the protease inhibitors and five times with PBS. The antigens were then eluted with SDS- or urea-containing sample buffers for one- or two-dimensional polyacrylamide gel analyses, respectively.

For affinity chromatography, bovine urothelium was isolated by scraping the bladder mucosa with a blunt scalpel, and washed twice with ice-cold PBS. The cells were collected by centrifugation at 1,500 g for 5 min, and then lysed with Triton X-100 lysis buffer. After centrifugation at 10,000 g at 4°C for 30 min, the supernatant was diluted with lysis buffer to 1 mg protein per ml. Affinity chromatography was performed batch-wise using 1 ml of antibody-Sepharose conjugates per 500 ml of the extract. The subsequent steps were essentially the same as the immunoprecipitation procedure except that the bound antigen was eluted with 1% octyl glucoside, 0.05 M diethylamine, pH 11.5. The eluates were dialyzed overnight against PBS and then concentrated with a microconcentrator (Centricon-10; Amicon Corp., Danvers, MA).

Gel Electrophoresis, Immunoblot, and Autoradiography

Affinity-purified proteins were dissolved in 1% SDS, 25 mM Tris-HCl (pH 7.4) and separated by SDS-PAGE according to Laemmli (1970), using a 15% polyacrylamide gel with an acrylamide/bisacrylamide ratio of 120:1. The apparent molecular weights of uroplakins were determined by comparing their electrophoretic mobilities with those of marker proteins with known sizes (Fig. 6 a, lane 4). Two-dimensional, nonequilibrium pH gradient gel electrophoresis (NEpHGE-SDS) was performed as described by O'Farrell et al. (1977). The separated proteins were electrophoretically transferred to nitrocellulose paper, stained with Fast Green, and then with antibodies using the peroxidase-anti-peroxidase method (Towbin et al., 1979; Woodcock-Mitchell et al., 1982; Eichner et al., 1984). For autoradiography, the gels were dried and exposed to Kodak XAR-5 film at room temperature.

Results

mAb AE31 Recognizes a Urothelial-specific, Maturation-related Surface Antigen

To identify and localize precisely the protein subunits of AUM, we initiated an effort to generate specific antibodies. Our first attempt consisted of partially purifying bovine AUM according to Caruthers and Bonneville (1977), isolating several major proteins from it by preparative SDS-PAGE, and using them to immunize rabbits and chickens. However, despite repeated injections no immunological responses could be elicited to most of these proteins suggesting that they must be extremely nonimmunogenic. This may explain why no prior AUM-specific antibody has been reported in the literature. As an alternative approach, we injected a crude bovine urothelial membrane fraction, without SDS denaturation, into Balb/c mice. After fusing the stimulated spleen cells with myeloma cells, we screened for monoclonal antibodies that produced specific immunofluorescent staining of the AUM-rich, urothelial superficial (umbrella) cells. Of the ∼1,000 clones that were screened, six showed strong stain-
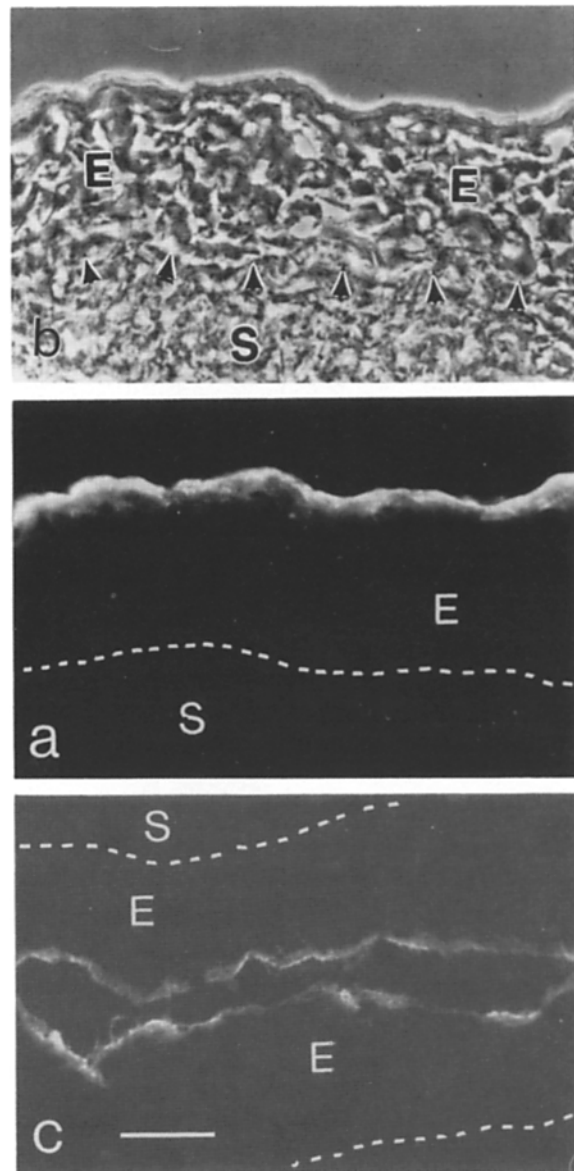

Figure 1. Immunofluorescent staining of mammalian urinary bladder sections with AE31 antibody. (a) AE31 staining of bovine bladder epithelium; b shows the phase-contrast image of the same field. (c) AE31 staining of human ureter epithelium. Control experiments using a nonrelevant mouse mAb produced by P3, an immunoglobulin-secreting myeloma cell line, produced no staining (not shown). E and S, epithelium and stroma, respectively. Arrowheads in a, as well as dashed lines in b and c, denote epithelial-stromal junction. Bar, 50 μm.

ing of umbrella cells (Figs. 1, a and b) with no detectable staining of cells in bovine skin, cornea, esophagus, kidney, liver, or muscle. One of these antibodies, designated AE31, exhibited high antigen avidity and crossreacted with urothelial umbrella cells of rabbit, monkey and human (Fig. 1 c), suggesting that its epitope must be highly conserved. Therefore this antibody was chosen for further characterization.

Immunofluorescent staining of cultured bovine urothelial cells revealed selective staining of some superficial cells that apparently had attained an advanced stage of differentiation
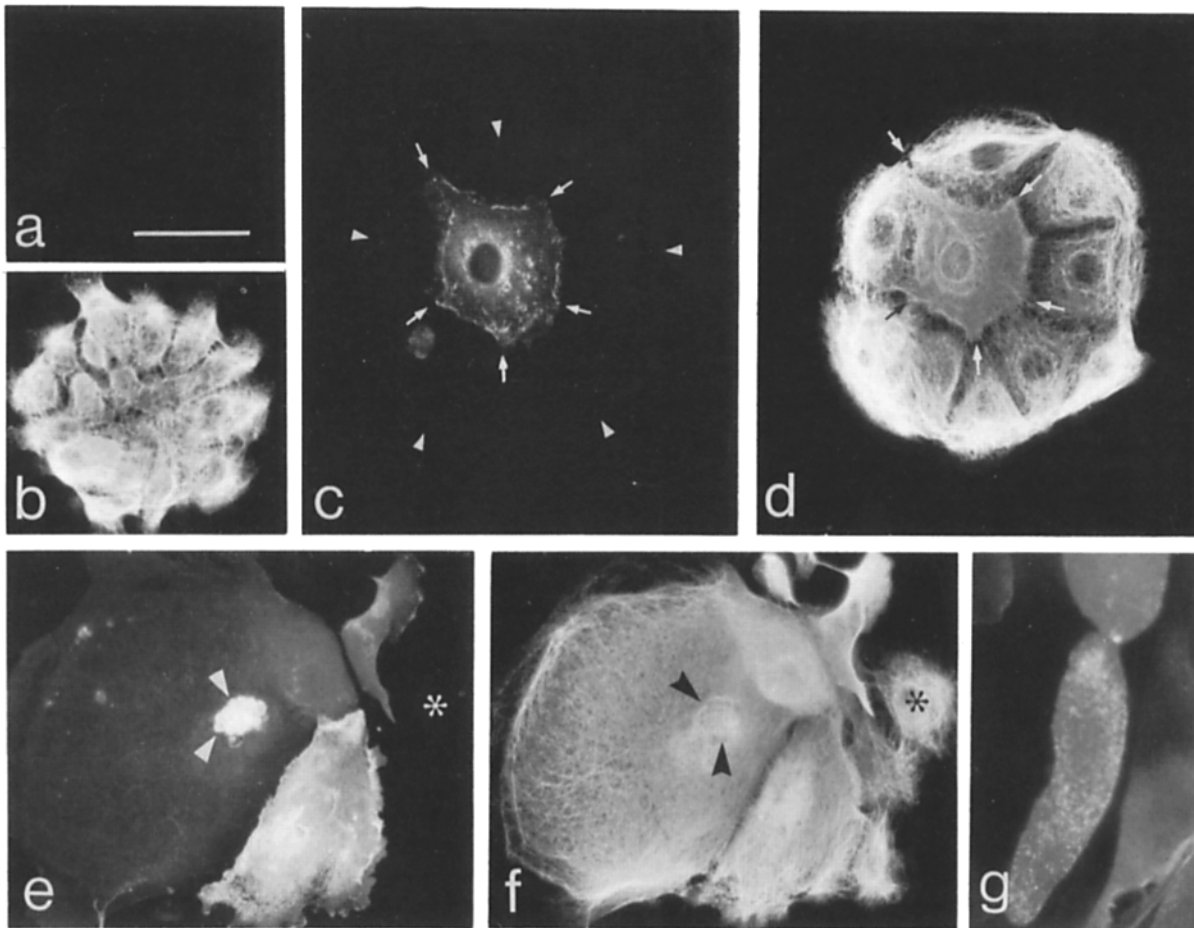
Figure 2. Immunofluorescent staining of cultured bovine urothelial cells with AE31 antibody. Cultured urothelial cells (3–10 d) were fixed and permeabilized with acetone-methanol (1:1), and double-stained with mouse AE31 (*a*, *c*, and *e*) and a rabbit antiserum to keratin (*b*, *d*, and *f*; corresponding fields of *a*, *c* and *e*, respectively). Arrowheads and arrows in *c* and *d* delineate the colony and an AE31-positive cell, respectively. In *e* and *f*, asterisks marks a keratin-positive, AE31-negative basal cell, whereas the arrowheads denote some AE31-positive cytoplasmic vesicles. Note the selective staining of a subpopulation of superficial cells by AE31. *g* shows the AE31 staining of a non-permeabilized culture (see Materials and Methods). Bar, 50 μm.

(Fig. 2, *a–f*). Because this staining does not require cell-permeabilization (Fig. 2 *g*), at least some of the AE31 epitope must be exposed on the apical cell surface. No staining was observed with cultured bovine epidermal cells, esophageal epithelial cells or fibroblasts (data not shown), thus confirming the urothelial specificity of the AE31 epitope.

AE31 Identifies a 27-kD Protein that Copurifies with a 15- and a 47-kD Protein

The bovine urothelial antigen(s) reacting with AE31 antibody were isolated in one step by affinity chromatography. Urothelial membrane proteins soluble in 1% Triton X-100 were passed through an AE31-conjugated affinity column. Two major proteins, one 27 and another 15 kD, were isolated in a roughly equimolar ratio (Fig. 3 *a*, lane *1*). A third, 47-kD protein was also routinely identified, although only in small and variable amounts (Fig. 3 *a*, lane *1*). The same three proteins were immunoprecipitated from [³⁵S]methionine-labeled, cultured bovine urothelial cells (Fig. 3 *b*, lane *1*; Fig. 4). These proteins were not precipitated, however, by a control antibody (P3, data not shown; also see Fig. 3 *a*,

lane *3*). Moreover, these three proteins appear to be urothelium-specific as they were not detected in cultured bovine esophageal epithelial cells (Fig. 3 *b*, lane *2*), epidermal cells (lane *3*) or bladder fibroblasts (lane *4*).

Since the same three affinity-purified proteins were observed after SDS-PAGE, with and without sample reduction, these proteins clearly are not disulfide cross-linked. Immunoblotting experiments further established that, of these three proteins, only the 27-kD species possesses the AE31 epitope (Fig. 3 *a*, lane 2). These results indicate that the 15- and 47-kD species must have been copurified through their noncovalent interactions with the 27-kD antigen.

Analyses of these proteins by two-dimensional polyacrylamide gel electrophoresis indicate that the 27-kD protein is slightly acidic (pI ∼5.5), whereas its associated 15-kD protein is slightly basic (pI ∼7.8; Fig. 4). The minor 47-kD protein comigrates with the 27-kD species during first dimensional charge separation (Fig. 4), suggesting that these two molecules either are isoelectric or, perhaps more likely judging from their identical smearing patterns, form a tight complex that survived the 9.5 M urea present in the first dimensional separating gel.
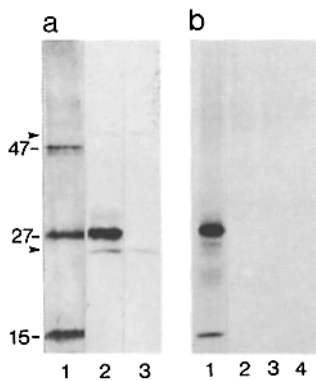
Figure 3. (a) Affinity purification and immunoblotting of AE31 antigens. AE31 antigens were purified from bovine bladder epithelium by affinity chromatography (see Materials and Methods), dissolved in 1% SDS, 25 mM Tris-HCl (pH 7.4) and analyzed by SDS-PAGE. The isolated proteins were (lane *1*) stained with Coomassie blue, (lane *2*) immunoblotted with AE31 antibody or (lane *3*) immunoblotted with P3 control antibody. Note in lane *1* that two major (27 and 15 kD) and one minor (47-kD) protein were purified, and in lane *2* that AE31 recognizes specifically the 27-kD species. *H* and *L* denote a small amount of (AE31) immunoglobulin heavy and light chains, respectively, that leaked from the affinity column and were detected by immunoblotting. (b) Immunoprecipitation of AE31 antigens from cultured bovine urothelial cells. Primary cultures of various bovine cells (80% confluent) were labeled with [³⁵S]methionine (0.2 mCi/2 ml per 100-mm dish) for 10 h. The Triton X-100 lysates were pretreated with P3-Sepharose conjugates before they were incubated with the AE31-Sepharose conjugates. The bound antigens were eluted with SDS sample buffer and analyzed by SDS-PAGE. Samples are from: lane *1*, urothelial cells; lane *2*, esophageal epithelial cells; lane *3*, epidermal cells; and lane *4*, bladder fibroblasts. Note the detection of the 27- and 15-kD proteins in urothelial cells, and their absence in other cells. The ratio of these two proteins appear to differ from lane *1* of Fig. 3, most likely reflecting different methionine contents. The 47-kD protein is barely visible in this gel but can be seen readily in Fig. 4, which shows the pattern of an overloaded two-dimensional polyacrylamide gel.
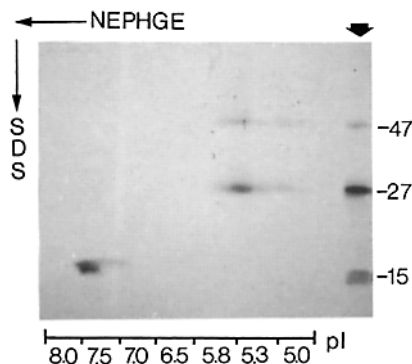

Figure 4. Analysis of AE31 antigens by two-dimensional polyacrylamide gel electrophoresis. [³⁵S]Methionine-labeled, AE31-reactive proteins were immunoprecipitated from cultured bovine urothelial cells and separated by two-dimensional gel electrophoresis. The horizontal and vertical arrows on the top left corner denote the directions of the first dimensional nonequilibrium-pH-gradient-electrophoresis (NEpHGE) and the second dimensional SDS-PAGE (SDS), respectively (O'Farrell et al., 1977). The heavy arrow on the upper-right corner denotes a side lane containing the same sample separated by SDS-PAGE only. The relative isoelectric point (*bottom*) were determined from a blank, first dimensional gel.

The 27- and 15-kD Proteins Are Immunologically Distinguishable

To determine whether the 27- and 15-kD proteins are related, we prepared a rabbit antiserum to a synthetic peptide corresponding to an NH₂-terminal amino acid sequence of the 15kD protein (data not shown). This antiserum was found
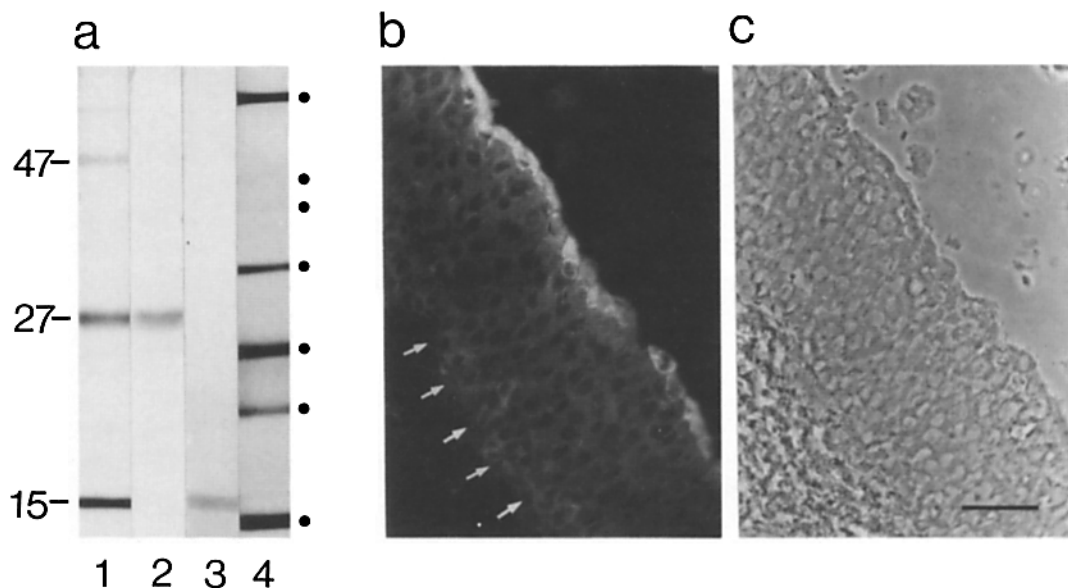

Figure 5. Characterization and immunolocalization of the 15-kD protein. (a) Lane *1*, urothelial proteins affinity-purified using AE31 antibody, as visualized by silver nitrate staining. Lane *2*, a sample as shown in lane *1* immunoblotted with AE31 antibody showing the selective staining of the 27-kD protein. Lane *3*, a similar sample as in lane *1* immunoblotted with a rabbit antiserum raised against a synthetic peptide corresponding to twelve amino acids located near the NH₂ terminus of the 15-kD protein; note its selective reaction with the 15-kD protein. Lane *4*, molecular weight standards (from the top, BSA, 66 kD; egg albumin, 45 kD; glyceraldehyde-3-phosphate dehydrogenase, 36 kD; carbonic anhydrase, 29 kD; trypsinogen, 24 kD; trypsin inhibitor, 20 kD; and lactalbumin, 14 kD). (b) Immunofluorescent staining of a frozen section of bovine urothelium using the antiserum to the 15-kD protein; arrows delineate the basement membrane zone. (c) Phase-contrast picture of the same field as in *b*. Note the selective staining of urothelial superficial cells. Bar, 50 μm.
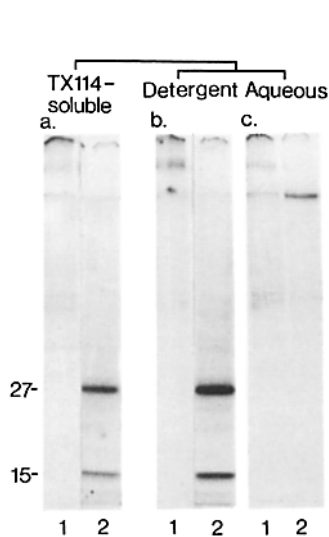
Figure 6. The 27- and 15-kD proteins remain in the detergent phase during Triton X-114 phase separation. [³⁵S]Methionine-labeled proteins were immunoprecipitated with AE31 (lanes 2) or P3 (lanes 1; control) antibodies, solubilized in 1% Triton X-114, and subjected to phase separation at 30°C as described by Bordier (1981). (a) Total Triton X-114 soluble fraction before phase separation, (b) the detergent fraction after phase separation, and (c) the aqueous fraction after phase separation. Note the exclusive distribution of the 27- and 15-kD proteins in the detergent phase.

Figure 7. Processing of the AE31 antigens as analyzed in a pulse-chase experiment. Nearly confluent cultures of bovine urothelial cells were pulse-labeled with [³⁵S]methionine for 10 min, and chased for various periods of time (marked in minutes on top). Triton X-100-soluble fractions of the cells were incubated with P3-Sepharose 4B conjugates (lanes *1, 3, 5, 7, 9,* and *11;* as controls), or AE31-Sepharose 4B conjugates (lanes *2, 4, 6, 8, 10,* and *12),* and the bound proteins were eluted and analyzed by SDS-PAGE. Note the initial appearance of a 32-kD protein which is processed, via a 30-kD intermediate, to a final mature form of 27-kD. Also note that the appearance of the coprecipitated 15-kD band coincides with that of the mature 27-kD band.

to react only with the 15-kD protein without crossreacting with the 27-kD protein (Fig. 5 *a,* lane *3).* This finding, together with the specificity of AE31 antibody for the 27-kD species (Fig. 5 *a,* lane *2),* indicate that these two polypeptides are immunologically distinct.

Immunofluorescent staining data indicate that the 15-kD protein, like the 27-kD protein, is also urothelium specific and can be localized specifically in the umbrella cells (Figs. 5, *b* and *c).*

The 27- and 15-kD Proteins Codistribute during Triton X-114 Phase Separation

It has been shown that when a Triton X-114 solution undergoes phase separation, many hydrophobic (integral) membrane proteins tend to stay in the detergent phase (Bordier, 1981). When [³⁵S]methionine-labeled and immunoprecipitated 27- and 15-kD proteins were subjected to such an analysis, both partitioned almost exclusively into the detergent phase (Figs. 6, *b* and *c).* This suggests that either both proteins are hydrophobic or, perhaps less likely, only one of them is highly hydrophobic and "carries" the other protein to the detergent phase through strong protein-protein interactions that remain stable in the detergent phase.

Processing of the 27-kD Molecule

In a pulse–chase experiment, cultured bovine urothelial cells were labeled for 10 min with [³⁵S]methionine and chased for various periods of time before immunoprecipitation with either AE31 (Fig. 7, *even-numbered lanes*) or P3 control (Fig. 7, *odd-numbered lanes).* The results clearly indicate that the newly synthesized AE31 antigen is a 32-kD precursor, which is later replaced by a 30-kD intermediate and finally by the mature 27-kD form. This maturation process, which presumably involves partial proteolysis, took 30–60 min to complete. Interestingly, the appearance of the 15-kD protein in the AE31 immunoprecipitates followed an almost identical time course, i.e., it appeared in significant amounts only after a chase of 30–60 min. Such kinetics have interesting implications on the maturation/interaction of the 27- and 15-kD molecules, and provide additional evidence that the 27-kD protein cannot be a precursor of the 15-kD molecule
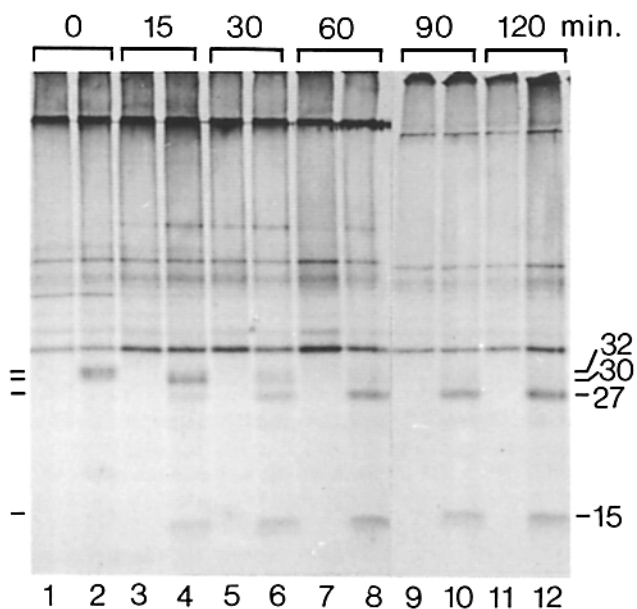
(see Discussion). These results also indicate that our cultured urothelial cells can not only synthesize but also process the 27-kD and, most likely, the 15-kD proteins.

The 27-kD Protein Is Associated with the Apical Surface of the AUM

To determine the ultrastructural location of the 27-kD antigen, we stained bovine urothelium sections with AE31 using a preembedding, immunogold labeling technique (Fig. 8). On the apical cell surface, strong staining was observed on the luminal leaflet of the asymmetric unit membrane without any staining of the cytoplasmic leaflet (Fig. 8 *b).* Such an asymmetric staining pattern is consistent with previous EM data showing that the 12-nm protein particles are associated exclusively with the apical leaflet without penetrating into the cytoplasmic leaflet (see Discussion).

Strong AE31 staining was also observed in numerous cytoplasmic vesicles presumably representing different stages of AUM-maturation (Fig. 8 *b).* In some small (immature) vesicles located deep in the cytoplasm near the Golgi apparatus, AE31 stains the cytoplasmic side sparingly and in a patchy fashion (arrowheads in Fig. 8 *b;* for higher magnification views, see Fig. 8, *c* and *d).* In most other vesicles, AE31 stains the cytoplasmic leaflet strongly and evenly (Fig. 8, *e* and *f).* Although in all these aforementioned vesicles no staining was found on the luminal leaflet, vesicles can be found showing AE31 staining of both the cytoplasmic and
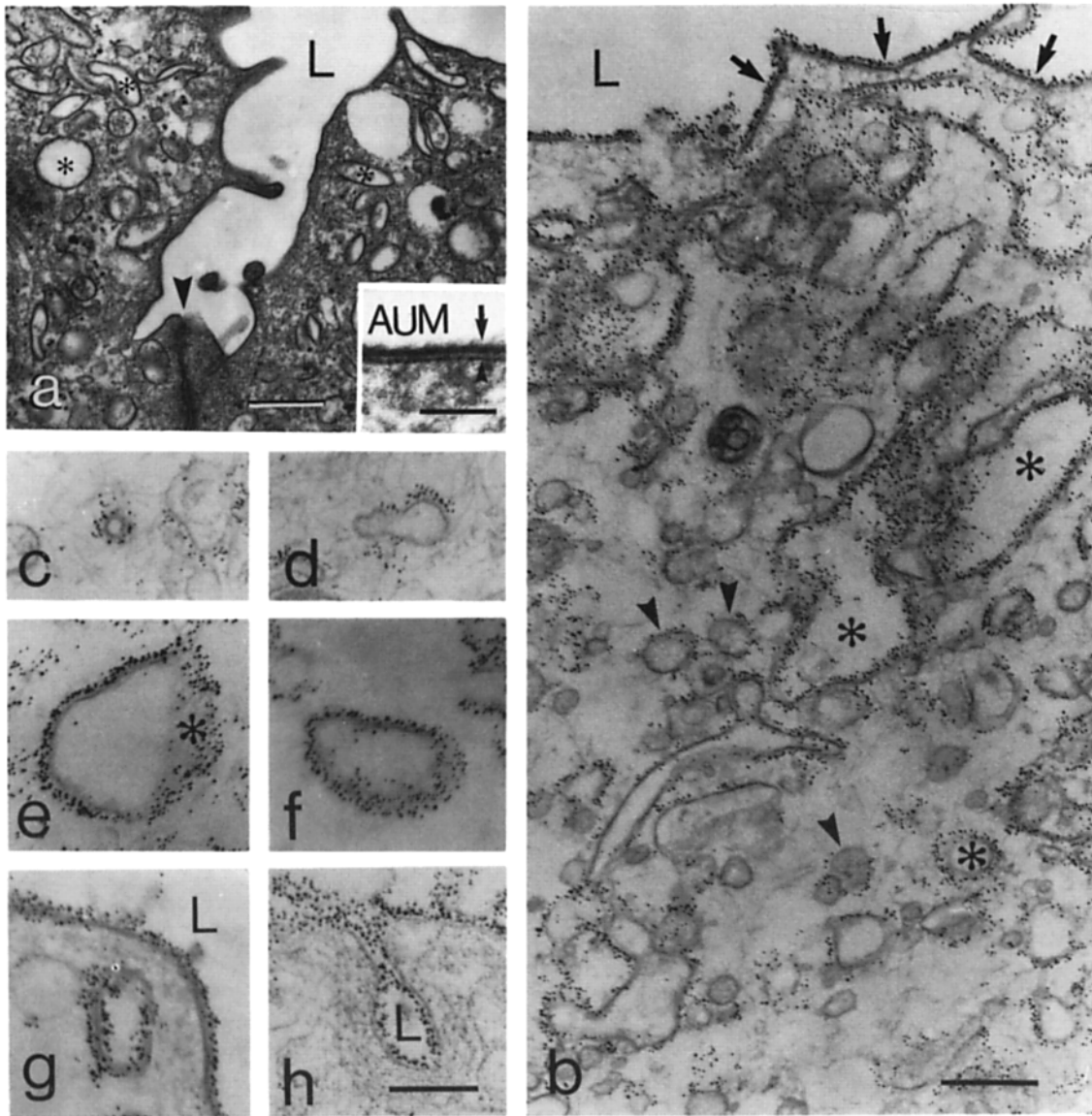
Figure 8. Ultrastructural localization of the 27 kD protein in urothelial superficial cells. (*a*) shows the ultrastructural appearance of two neighboring urothelial superficial (umbrella) cells. Note numerous fusiform or discoidal vesicles (*asterisks*), the thick asymmetric unit membrane (AUM; shown at higher magnification in the insert) facing the lumen (*L*), and a junctional complex (*arrowhead*). (*b*) A survey picture showing the immunogold labeling of an umbrella cell with AE31 antibody using a preembedding method. Note the strong labeling of the luminal leaflet of the AUM lining the apical surface (arrows in the luminal space); the labeling of some vesicles in the submembranous area indicates that this area is accessible to antibody molecules. Also note that some of the small vesicles (*arrowheads*) were labeled in patches on the cytoplasmic side, while some other vesicles (*asterisks*) were labeled on both cytoplasmic and luminal sides. Selected examples demonstrating different AE31 staining patterns of cytoplasmic vesicles, presumably reflecting an increasing degree of vesicular maturation, are shown from *c* to (*h*). Note the patchy staining of the cytoplasmic side in some early vesicles (*c* and *d*), the staining of both sides in some vesicles presumably representing intermediate stages of maturation (*e* and *f*; asterisk in panel *e* denotes a tangential cut of the membrane), and the exclusive staining of the luminal side of the mature, apical surface (*g* and *h*). Also note the presence of some unlabeled cytoplasmic vesicles presumably representing lysosomes and mitochondria. Bars: (*a*) 0.3 μm; (*inset* of *a*); 0.02 μm; (*b*) 0.2 μm; (*c–h*) 0.15 μm.

luminal sides (Fig. 8, *f* and *g*). Finally, in a few vesicles located immediately underneath (some obviously have already fused with) the apical surface, the labeling pattern was consistent with that of the mature plaques, i.e., limited to the luminal leaflet (arrows in Fig. 8 *b*; for higher magnification views, see Fig. 8, *g* and *h*). Although we cannot rule out the possibility that the lack of luminal staining in some vesicles is due to inaccessibility of the antibody or to antigenic masking, the positive staining of the cytoplasmic surface strongly suggests that the AE31 epitope is present on this side in a great majority of vesicles. This raises the interesting possibility that AE31 epitope may be transferred from the cytoplasmic side of the membrane to the luminal side during a final stage of plaque maturation (see Discussion).

Discussion

A major obstacle in studying the AUM has been the inability to generate specific antibodies to any of its putative protein subunits. Such antibodies are of obvious importance not only for immunolocalization but also for cDNA library screening. Having failed to raise antisera to AUM by immunizing animals with SDS-denatured proteins, we decided to use as our immunogen native, partially purified urothelial membrane for monoclonal antibody production. The rationale for this approach was that the highly regular, polyvalent, hexagonal arrays of proteins exposed on the plaque surface may be favorably presented and, hence, more immunogenic (Dintzis and Dintzis, 1988). Moreover, the hybridoma approach coupled with a highly specific and sensitive assay allows us to obtain even minor antibody species. This approach indeed worked well, and yielded the AE31 antibody with which we have been able to identify a 27-kD AUM protein subunit and two of its associated proteins (15 and 47 kD).

Existence of Three AUM-related Proteins

Using the AE31 antibody as a probe, we have identified a 27-kD bovine urothelial membrane protein. Although proteins in the same size range have previously been detected in AUM preparations of pig (25–27 kD; Vergara et al., 1974), sheep (33 kD; Caruthers and Bonneville, 1977) and cow (22 kD; Stubbs et al., 1979), little was known about their tissue distribution, their relationships with other proteins found in the same AUM preparations, and, most importantly, whether they are AUM-associated in situ. Our data indicate that the 27kD bovine protein is urothelium-specific (Figs. 1 and 3 *b*), is expressed only during an advanced stage of urothelial differentiation in the AUM-rich umbrella cells (Figs. 1, 2, and 8), and can be immunolocalized to the apical surface of mature AUM (Fig. 8). Thus compelling evidence is now available establishing that this 27-kD protein is a marker for an advanced stage of urothelial differentiation and is AUM-associated in situ. Based on its association with *urothelial plaques* and anticipating more proteins in this class, we named this 27-kD protein uroplakin I.

Reasonable evidence has also become available implicating a 15-kD protein as another major AUM subunit. Although low molecular weight proteins have been noted in some prior AUM preparations, these proteins were largely ignored because they were present only in minute quantities (possibly due to proteolysis as no protease inhibitors were used in these earlier studies). We identified the 15-kD protein through its strong interactions with the 27-kD AE31 antigen. These two proteins not only copurify in roughly equimolar ratio during affinity chromatography (Fig. 3 *a*), but also codistribute during Triton X-114 phase separation (Fig. 6). Immunofluorescence staining using an antiserum to the 15-kD protein has established that this 15-kD protein colocalizes with the 27-kD protein in urothelial superficial cells (Fig. 5). Together, these data strongly imply that the 15-kD protein interacts in a 1:1 molar ratio with the 27-kD uroplakin I to form the AUM. For this reason, we provisionally designate this protein as uroplakin II—although unambiguous demonstration of its plaque association in situ has not yet been established.

A third, 47-kD protein was also reproducibly identified, although in relatively small and variable quantities, in our affinity-purified, AE31 antigen preparations (Figs. 3 *a* and 4). This protein probably corresponds to the minor 55-kD component in Vergara's preparation (1974) and the 41–62-kD minor component in Caruthers and Bonneville's preparation (1977). Although it was speculated that this high–molecular weight component represents a dimer of the (25–)27-kD protein (Vergara et al., 1974), the fact that none of our antibodies to the 27- and 15-kD molecules crossreact with the 47-kD molecule (Figs. 3 *a* and 5) indicates that the 47-kD protein must be immunologically distinct. Whether this 47-kD protein is truly a part of AUM remains to be established.

Although our data cannot rule out additional plaque-associated proteins, we found recently that a highly purified preparation of bovine urothelial plaques gave rise to (only) three major proteins that comigrated precisely during SDS-PAGE with the three affinity-purified (15, 27, and 47 kD) proteins (Wu, X.-R., et al., manuscript in preparation). Moreover, in light of our current findings it has become apparent that these three proteins or their equivalents are also present in urothelial plaques of both pig (Vergara et al., 1974) and sheep (Caruthers and Bonneville, 1977). Together, these data raise the possibility that the three urothelial proteins that we describe in this paper represent the major polypeptide constituents of bovine urothelial plaques.

Based on the earlier observation that a major protein in the molecular weight range of 27–33 kD is present in sheep and pig AUM preparations, it has been suggested that all 12 inner and outer subunits of the protein particle of AUM are composed of this protein (Taylor and Robertson, 1984). An important implication of this is that AUM is a homopolymer. Our present finding of multiple AUM-associated and -related proteins raises the interesting possibility that AUM may actually be a heteropolymer composed of multiple protein subunits.

Synthesis and Assembly of Uroplakins

Freeze fracturing and transmission electron microscopy studies of the fusiform vesicles at different stages of maturation have shown that few, if any, 12 nm protein particles are present in the Golgi apparatus or in the immature vesicles that just bud off from it (Severs and Hicks, 1979; Alroy et al., 1982). However, the density and the degree of packing of these particles increase progressively upon vesicle maturation, culminating in the formation of large areas of semi-crystalline, hexagonally packed particles in mature plaques (Severs and Hicks, 1979). These findings suggest that the 12-nm particles are formed mainly during an advanced, post-Golgi stage of AUM maturation, possibly involving the insertion of plaque-associated proteins synthesized by cytoplasmic polysomes congregating near the forming face of the Golgi apparatus (Severs and Hicks, 1979; Alroy et al., 1982). Obviously, these existing data cannot rule out the possibility that some of the protein subunits may actually be present in the immature vesicles but somehow are unable to assemble into morphologically recognizable 12-nm particles. In this regard, it is significant that our AE31 antibody produces weak and patchy staining in some immature vesicles but the staining becomes progressively stronger and more extensive upon vesicle maturation (Fig. 8, *d–i*; see below). Although the possibility of antigenic masking/unmasking remains, the correlation between AE31 staining intensity and the morpho-

genesis of particle-containing vesicles suggests that the AE31 antigen is present only in small amounts in immature vesicles but is accumulated upon vesicle maturation.

By the immunogold technique, the AE31 epitope of the 27-kD protein can be localized on the luminal side of apical plaques in association with the thickened outer leaflet (Fig. 8, *d*, *m* and *n*). Few, if any, gold particles are detected on the cytoplasmic side of these plaques. Because some vesicles immediately beneath the plasma membrane are strongly labeled (Fig. 8 *d*), this lack of staining is not due to incomplete antibody penetration. Such a localization of the AE31 antigen on the outer leaflet of AUM is entirely consistent with our immunofluorescent staining data showing that the epitope is exposed on the cell surface (Fig. 2 *g*), as well as earlier electron microscopic data indicating that in mature plaques the subunit proteins are largely limited to the outer leaflet without penetrating to the cytoplasmic surface (Staehelin et al., 1972; Knutton and Robertson, 1976; Brisson and Wade, 1983; Taylor and Robertson, 1984).

Different AE31 staining patterns are observed in some cytoplasmic vesicles located deep in the cytoplasm, presumably representing earlier stages of plaque maturation (however, see Severs and Hicks, 1979). Some of these vesicles show patchy labeling of the cytoplasmic leaflet (Fig. 8, *c* and *d*), whereas others show labeling of both cytoplasmic and luminal sides (Fig. 8, *f* and *g*). Those vesicles located immediately underneath, or have just fused with, the apical cell surface, show staining of only the luminal surface (Fig. 8 *h*). These results strongly suggest that in immature vesicles at least some of the AE31 epitope (of the 27-kD protein) must reside on the cytoplasmic side, but is later translocated to the luminal side as the vesicles mature and eventually fuse with the apical surface. Such a translocational event may explain some earlier TEM data indicating that the polarity of some of the immature vesicles appears to be reversed, i.e., they have a thickened cytoplasmic, rather than luminal, leaflet (Alroy et al., 1982). More data are needed to test this interpretation and, if substantiated, to unravel the mechanisms of this most unusual phenomenon. With the availability of specific antibodies and a culture system capable of synthesizing and processing plaque-related proteins, we are in a good position to study this and other events related to the biosynthesis and maturation of AUM.

Processing of Uroplakins I and II

Pulse–chase experiments indicate that the 27-kD uroplakin I is first synthesized as a 32-kD precursor, which is processed to a 30-kD intermediate before finally maturing into 27 kD (Fig. 7). It is possible that some of these decreases in size are related to the removal of signal peptides involved in targeted protein transport and/or translocation during AUM biogenesis (Sabatini et al., 1982; Duffaud et al., 1985; Rapport and Wiedmann, 1985; Pfeffer and Rothman, 1987; Rose and Doms, 1988; Gennis, 1989; Randall and Hardy, 1989).

The behavior of the 15-kD uroplakin II during the pulse–chase experiment (Fig. 7) is unexpected in that it does not immunoprecipitate until the cells have been chased for ∼30 min when the mature 27-kD protein begins to appear. This raises the possibility that the 15-kD protein may be first synthesized as a precursor that is unable to interact with the mature (unlabeled) 27-kD protein, and is therefore not detectable by immunoprecipitation using AE31.

Concluding Remarks

Although the ultrastructure of AUM has been studied in great detail allowing the construction of three-dimensional models with 30-A° resolution (Brisson and Wade, 1983; Taylor and Robertson, 1984), significant confusion remained regarding its protein composition. In this paper, we have identified two major (27- and 15-kD) uroplakins and one minor 47-kD associated protein. Moreover, EM localization data indicate that an epitope of uroplakin I undergoes an unusual form of translocation during plaque maturation. The identification of these three plaque-related molecules clarifies some seemingly conflicting data existing in the literature, and paves a way for studying the structural and functional roles of these proteins during normal and abnormal urothelial differentiation.

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