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BJ Cell

Biochem. J. (2008) **414**, 195–203 (Printed in Great Britain) doi:10.1042/BJ20080550

Assembly of a membrane receptor complex: roles of the uroplakin II prosequence in regulating uroplakin bacterial receptor oligomerization

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The apical surface of the mammalian urothelium is almost completely covered by two-dimensional protein crystals (known as urothelial plaques) of hexagonally packed 16 nm particles consisting of two UP (uroplakin) heterodimers, i.e. UPs Ia/II and Ib/III pairs. UPs are functionally important as they contribute to the urothelial permeability barrier function, and UPIa may serve as the receptor for the uropathogenic *Escherichia coli* that causes over 90 % of urinary tract infections. We study here how the UP proteins are assembled and targeted to the urothelial apical surface, paying special attention to the roles of the prosequence of UPII in UP oligomerization. We show that (i) the formation of the UPIa/UPII heterodimer, necessary for ER (endoplasmic reticulum) exit, requires disulfide formation in the prosequence domain of proUPII (the immature form of UPII still containing its prosequence); (ii) differentiation-dependent N-glycosylation of the prosequence leads to UP stabilization; (iii) a failure to form tetramers in cultured urothelial cells, in part due to altered glycosylation of the prosequence, may block two-dimensional crystal formation; and (iv) the prosequence of UPII remains attached to the mature protein complex on the urothelial apical surface even after it has been cleaved by the *trans*-Golgi-network-associated furin. Our results indicate that proper secondary modifications of the prosequence of UPII play important roles in regulating the oligomerization and function of the UP protein complex.

Key words: disulfide formation, glycosylation, integral membrane protein, prosequence, protein assembly, uroplakin.

INTRODUCTION

The urothelial plaques that cover almost the entire mammalian urothelial apical surface provide a unique model system for studying the mechanisms and regulation of membrane assembly. Urothelial plaques [also known as AUM (asymmetric unit membrane)] consist of hexagonally packed two-dimensional crystals of 16 nm protein particles [1–3]. The urothelial plaques are composed of four major integral membrane proteins, i.e. UPs (uroplakins) Ia, Ib, II and IIIa [4–7]. UPIa (27 kDa) and UPIb (28 kDa) are closely related, sharing ~40 % of their amino acid sequences. They have four transmembrane domains and belong to the tetraspanin superfamily, which includes CD9, CD63, CD81, CD82 and CD151, that are known to play roles in important cellular functions including fertilization, immunological signalling and viral infection [7–16]. UPII and UPIIIa are related to each other as they share a stretch of ~12 amino acid residues in the extracellular, juxtamembrane position, and feature only a single transmembrane domain [6,17,18]. UPII is synthesized as a precursor containing an N-terminal presequence (26 amino acids) and a prosequence (59 amino acids) followed by the mature protein (100 amino acids; [17]). The mature UPII (15 kDa) consists of a long extracellular domain of 71 amino acids and a transmembrane domain of 25 amino acids, with very little intracellular domain [17]. UPIIIa (47 kDa) is also synthesized as a pre-protein; the mature protein consists of a polypeptide of ~29 kDa plus ~18 kDa equivalents of complex glycans, and it is the only UP that has a significant cytoplasmic domain of ~52 amino acid residues [6].

It is important to study the structure and function of UPs, because (i) UP knockout leads to the loss of urothelial plaques and to a diminished urothelial permeability barrier function [19,20], and (ii) the high-mannose glycan of UPIa may serve as the receptor for type 1-fimbriated *Escherichia coli* that is the predominant causative agent for urinary tract infections [21,22]. Since urothelial umbrella cells are filled with UP-delivering vesicles that can be induced to fuse with the apical surface [23–26], such cells provide unique opportunities for studying how UPs are synthesized, assembled and targeted to epithelial apical compartments.

Much has been learned recently about the assembly of UPs. Existing data indicate that UPIa and UPIb interact with proUPII (the immature form of UPII still containing its prosequence) and UPIIIa respectively to form UPIa/UPII and UPIb/UPIIIa heterodimers that can then exit from the ER (endoplasmic reticulum) [18,27–29]. We have shown previously that a monoclonal antibody, AE31, recognizes a composite proUPII epitope consisting of a segment of the prosequence (−50 to −40) and another of the mature sequence (+40 to +60), suggesting that proUPII adopts a hairpin-like structure [28]. The use of AE31

Abbreviations used: asterisks indicate the glycosylated forms of each protein; AUM, asymmetric unit membrane; BN-PAGE, blue native PAGE; DMEM, Dulbecco's modified Eagle's medium; DSS, disuccinimidyl suberate; DTT, dithiothreitol; endo F, endoglycosidase F; endo H, endoglycosidase H; ER, endoplasmic reticulum; FBS, fetal bovine serum; TGN, *trans*-Golgi network; UP, uroplakin; proUPII, the immature form of UPII still containing its prosequence; pro-UPII, proUPII in which the prosequence has been cleaved by furin, but remains attached to UPII; UPIa, UPIb, UPII and UPIIIa, the four principal UP proteins.

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and other antibodies to conformation-dependent UP epitopes showed that the UPIa-dependent, furin-mediated cleavage of the prosequence of proUPII leads to global conformational changes of the mature UPII, and that UPIb also induces conformational changes in UPIIIa [28]. We also demonstrated that proUPII, which harbours two cysteine residues and three N-glycosylation sites in its prosequence, undergoes differentiation-dependent glycosylation; we proposed that defects in the glycosylation of proUPII may block tetramer formation in cultured urothelial cells, thus explaining the lack of two-dimensional crystal formation in these cells [28]. These observations suggest that the prosequence of proUPII is involved in modulating UP assembly. However, the mechanism by which the prosequence influences UP assembly, and the fate of the prosequence after it is cleaved by the TGN (*trans*-Golgi network)-associated furin, remain unclear.

In the present paper, we evaluate the functional roles of the UPII prosequence in UP assembly by mutagenesis coupled with transfection studies. Our results indicate that proper glycosylation and disulfide bond formation in the UPII prosequence are critical for proUPII to interact with its partner UPIa and be processed to mature UPII. Moreover, we demonstrate that the UPII prosequence remains associated with the mature UPII even after it has been cleaved by furin, and that defective glycosylation of proUPII in cultured urothelial cells may block the formation of UP heterotetramers, thus explaining the failure of UPs to assemble into two-dimensional crystals in such cells.

MATERIALS AND METHODS

All reagents were obtained from our usual sources [28]; DTT (dithiothreitol) and tunicamycin were purchased from Calbiochem; DSS (disuccinimidyl suberate) from Pierce; and dialysed serum from Gibco BRL. Crude membranes and urothelial plaques [AUM (asymmetric unit membrane)] from bovine bladders were prepared via sucrose cushion centrifugation and Sarkosyl washing, as described previously [30]. The proteins were analysed by SDS/PAGE (17 % polyacrylamide) and immunoblotted as described in [30].

For cloning, all cDNAs used were full length, i.e. including the pre-, pro- and mature sequences. The terms 'proUPII' and 'pro-UPII' are used to indicate whether the furin-cleavage site is intact or cleaved respectively. Mutagenesis was performed according to the manufacturer's instructions using the QuikChange® site-directed mutagenesis kit (Stratagene). The sense oligonucleotides used for the N-glycosylation sites were, with the altered codons underlined: 5′-TGTCCGGAGAGCCCAGGACAGCA-AAGTGGTG-3′ (−19 N/Q); 5′-CCTCACAGGGGGCCAGGC-CACACTGACTG-3′ (−28 N/Q); and 5′-GCTGCAGCTGACT-TCCAGATCTCAAGCCTCTCTGG-3′ (−57 N/Q). For the two cysteine residues we used: 5′-GGTGCCTCCGAGCCGCGGA-CGCA-3′ (−5 C/S); and 5′-GCCTTGCCCCCAAGTCACCT-CACAGGG-3′ (−34 C/S). Plasmid DNA constructs were amplified using Qiagen minipreps and confirmed by DNA sequencing. Inserts were excised, purified using an agarose-gel system, and re-ligated into the original pcDNA3 vector as described previously [28].

Bovine urothelial cells were grown in DMEM (Dulbecco's modified Eagle's medium) supplemented with 15 % (v/v) FBS (fetal bovine serum) in the presence of mitomycin C-treated 3T3 feeder cells, as described in [31]. COS-1 cells (A.T.C.C.) were grown in DMEM containing 10 % (v/v) FBS and transfected as described previously [28,29] using FuGENE™ 6 (Roche), as per the manufacturer's instructions, and were analysed 24–48 h later. To prepare the lysates, cells were washed twice with PBS, pelleted, resuspended in the RIPA buffer [28] containing protease inhibitors (Cocktail Set 1; Calbiochem), rocked at 4 °C for 1 h, and centrifuged at 16000 ***g*** for 10 min at 4 °C. Cross-linking and immunoblotting were performed as described previously [28]. Equal loading of proteins was achieved by the BCA (bicinchoninic acid) protein quantification assay followed by staining nitrocellulose membranes using Ponceau S Red after protein transfer.

Pulse–chase labelling of transfected COS-1 cells using [^{35}S]methionine and [^{35}S]cysteine, as well as immunoprecipitation of labelled lysates using antibodies and Protein A/G–agarose beads, was performed as described previously [28,30]. Enzymatic deglycosylation of labelled lysates using endo H (endoglycosidase H) and endo F (endoglycosidase F) (New England Biolabs) was performed as described in [28,29].

Continuous polyacrylamide gradient blue native gels (5–18 % polyacrylamide) were prepared and overlaid with a 5 % polyacrylamide gel using previously described methods [32,33]. Samples of bovine AUM or crude membranes isolated from cultured bovine urothelial cells were made to contain 750 mM 6-aminocaproic acid, 50 mM Bis/Tris/HCl (pH 7.0), 1.25 % dodecylmaltoside and 0.35 % Coomassie Brilliant Blue G-250. Blue cathode buffer (50 mM Tricine, 15 mM Bis/Tris/HCl, pH 7.0, and 0.02 % Coomassie Brilliant Blue G-250) and anode buffer (50 mM Bis/Tris/HCl, pH 7.0) were used throughout. Protein bands on the gel were visualized by de-staining (25 % methanol and 10 % acetic acid), or gel lanes were cut into strips for the second dimension, regular SDS/PAGE.

For immunofluorescent staining, cultured cells were fixed for 20 min using 4 % (w/v) paraformaldehyde in PBS. Cells were blocked using 3 % (w/v) BSA, incubated with mouse monoclonal AE31 and/or rabbit anti-UPIa antibodies, and then incubated with FITC-conjugated donkey anti-mouse IgG and Texas Red-conjugated donkey anti-rabbit IgG antibodies.

RESULTS

Defining the conformation of proUPII using a panel of antibodies

As mentioned above, bovine UPII contains several domains: a presequence that is removed co-translationally, a prosequence (59 amino acid residues) harbouring three potential N-glycosylation sites and two cysteine residues, and a mature protein core (100 amino acids) containing a C-terminal transmembrane domain (Figure 1a). To study the immunoreactivity of various domains of the proUPII protein, we expressed UPII in COS-1 cells and performed immunoprecipitation using several antibodies to proUPII. The AE31 monoclonal antibody (Figure 1b, lane 2) pulled down ten times more proUPII than rabbit antibodies to the prosequence (lane 5) or to the synthetic peptide P4 of the mature UPII (lane 8), suggesting that the AE31 epitope (consisting of the amino acid residues −50 to −40 of the prosequence and residues +40 to +60 of the mature protein; [28,29]) is more strongly recognized than those defined by the other antibodies (see the Discussion section). Similar results were obtained when the cells were co-transfected with proUPII and UPIa, except that in this case, much of the proUPII had been processed to mature UPII (Figure 1b, lane 3; Figure 1d). A comparison of the four antibodies to P1–P4 epitopes showed that the immunoreactivities of P3 and P4 are more than ten times greater than those of P1 and P2 (Figure 1c). Each protein band identity was confirmed by means of deglycosylation and using different radiolabelling reagents ([^{35}S]methionine versus [^{35}S]cysteine) (Figure 1d). Taken together, these results indicate that the immunoreactivity of proUPII in immunoprecipitation assays decreases in the order of: AE31 $\gg$ prosequence $\approx$ P3 $\approx$ P4 $\gg$ P1 $\approx$ P2.

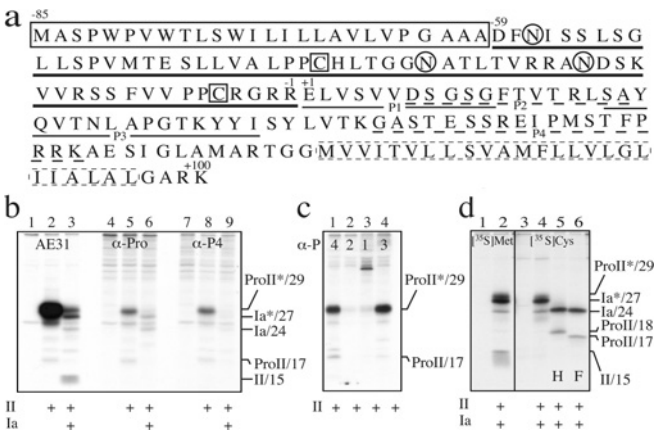

Figure 1 The AE31 antibody recognizes the 29 kDa proUPII much more strongly than antibodies to other proUPII epitopes

(**a**) The amino acid sequence of bovine proUPII, showing the presequence (boxed); prosequence (thick underlined) harbouring two cysteine residues (boxed) and three N-glycosylation motifs (circled); and the mature sequence (positions +1–100) containing the four synthetic peptide epitopes (P1–P4), and the single transmembrane domain (approximate location in dashed box). (**b**) Immunoprecipitation of proUPII. COS-1 cells were transfected with an empty pcDNA3 (lanes 1, 4 and 7), UPII cDNA, or UPII plus UPIa cDNAs (as indicated), [^{35}S]methionine-labelled and then immunoprecipitated using three different antibodies. Note the particularly strong reaction of AE31 with the 29 kDa proUPII, and, in lane 3, the co-immunoprecipitation of the 29 kDa proUPII with the 27 kDa UPIa and 15 kDa UPII; Ia/24 represents the non-glycosylated form of Ia*/27. (**c**) Immunoprecipitation of proUPII by antibodies to epitopes P1–P4 of the mature UPII peptide. (**d**) Differential labelling of the 15 kDa mature UPII. COS-1 cells were transfected with the pcDNA3 control plasmid (lanes 1 and 3) or UPII plus UPIa cDNAs, [^{35}S]methionine- (lanes 1 and 2) or [^{35}S]cysteine- (lanes 3–6) labelled and immunoprecipitated using the AE31 antibody. Before SDS/PAGE, samples in lanes 5 and 6 were treated with endo H and endo F respectively. Note the presence of methionine but not cysteine in the 15 kDa UPII band, confirming that this band is mature UPII. Results shown in (**b**–**d**) are representative of three independent experiments.

Disulfide linkage is important for maintaining a proper proUPII conformation and for UPII maturation

The proUPII contains only two cysteine residues, both residing in the prosequence (boxed in Figure 1a). To determine the possible roles of these two cysteine moieties in maintaining proper proUPII conformation, we performed several experiments. First, we studied the immunoreactivities of intact proUPII and various UPII mutants, in which one or both of the cysteine residues had been mutated to serine, using antibodies against various proUPII epitopes (Figure 2a). The results indicated that while cysteine mutations reduced the immunoreactivity of proUPII with AE31 ~2–3-fold (Figure 2a, lanes 1–5), they increased the proUPII immunoreactivities >10-fold for all other antibodies including those to the prosequence (lanes 6–10), and those to epitopes P1–P4 of the mature UPII (lanes 11–30). A similar increase in the immunoreactivity of proUPII as assessed using the anti-prosequence antibody was observed by Western-blot analysis of 2-mercaptoethanol-reduced proUPII after SDS/PAGE (Figure 2b). These results, in conjunction with our previous data [28], indicate that the conformation of proUPII is essentially SDS-resistant. To study the roles of disulfide linkage of the prosequence on proUPII maturation, we incubated COS-1 cells expressing UPII alone, or UPII plus UPIa in a culture medium containing 5 mM DTT; previous studies have shown that under this condition DTT can diffuse into the cells, resulting in the reduction of cellular proteins [34–37]. This led to a ~4-fold reduction in the processing of proUPII to mature UPII (Figure 3a). In addition, we studied the effects of cysteine-to-serine mutations (Figures 3b–3d). Immunoblot results clearly indicated that all such mutations blocked proUPII maturation (Figure 3b). A

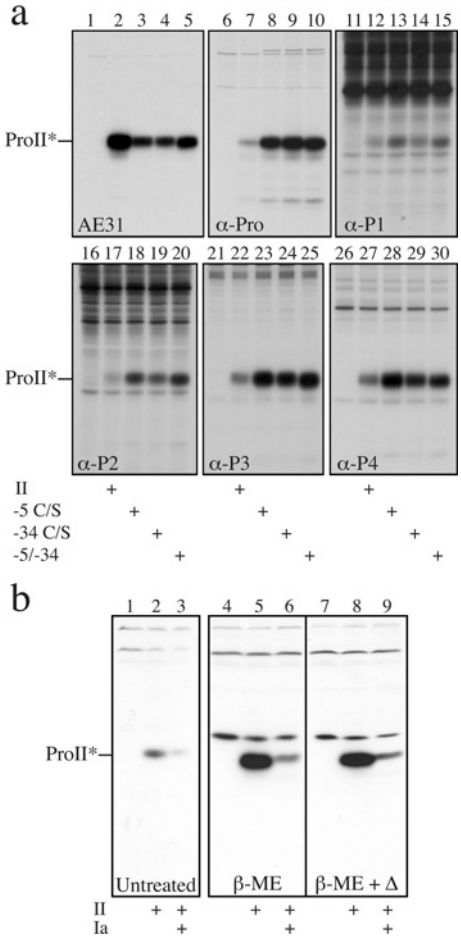

Figure 2 Loss of disulfide bonds leads to major changes in the immunoreactivities of proUPII

(**a**) Lysates of the [^{35}S]methionine-labelled COS-1 cells transfected with the empty pcDNA3 plasmid (lanes 1, 6, 11, 16, 21 and 26), or cDNAs encoding UPII, or various UPII cysteine mutants in which Cys^{-5} and/or Cys^{-34} in the prosequence were replaced by serine (as indicated), and immunoprecipitated using antibodies against various epitopes of proUPII. Note that disruption of the disulfide linkage leads to a decreased recognition of the proUPII by AE31, but to an increased reactivity by antibodies to all other epitopes. (**b**) Effects of 2-mercaptoethanol (2-ME) on the immunoreactivities of proUPII towards antibodies against the prosequence. COS-1 cells were transfected with an empty pcDNA3 plasmid (lanes 1, 4 and 7), UPII cDNA or UPII plus UPIa cDNAs (as indicated), and immunoblotted using an anti-prosequence serum. Some of the cell lysates were treated with 2-ME (3%, v/v; β-ME) (lanes 4–6) or with 2-ME plus boiling (β-ME + Δ) for 5 min (lanes 7–9). Lanes 1–3 are from the same gel as lanes 4–9, but were separated to avoid the reducing effect of 2-ME. Note the greatly increased immunoreactivity of the reduced 29 kDa band with antibodies against the prosequence. Results shown in each panel are representative of three independent experiments.

general decrease in proUPII in all co-transfectants suggested a competition for ribosomes from UPIa (Figure 3b, lanes 3, 5, 7 and 9). Immunoprecipitation of proUPII using the AE31 antibody showed that the cysteine-to-serine mutation decreased the amounts of co-immunoprecipitated UPIa by ~8–10-fold (Figures 3c–3d) in UPII/UPIa-co-expressing COS-1 cells, and resulted in an accelerated degradation of proUPII in UPII-singly-expressing cells (Figures 3e and 3f).

N-glycosylation is important for the proper conformation and stability of the proUPII

To test whether N-glycosylation plays a role in maintaining the ProUPII conformation, we mutated the three N-glycosylation sites

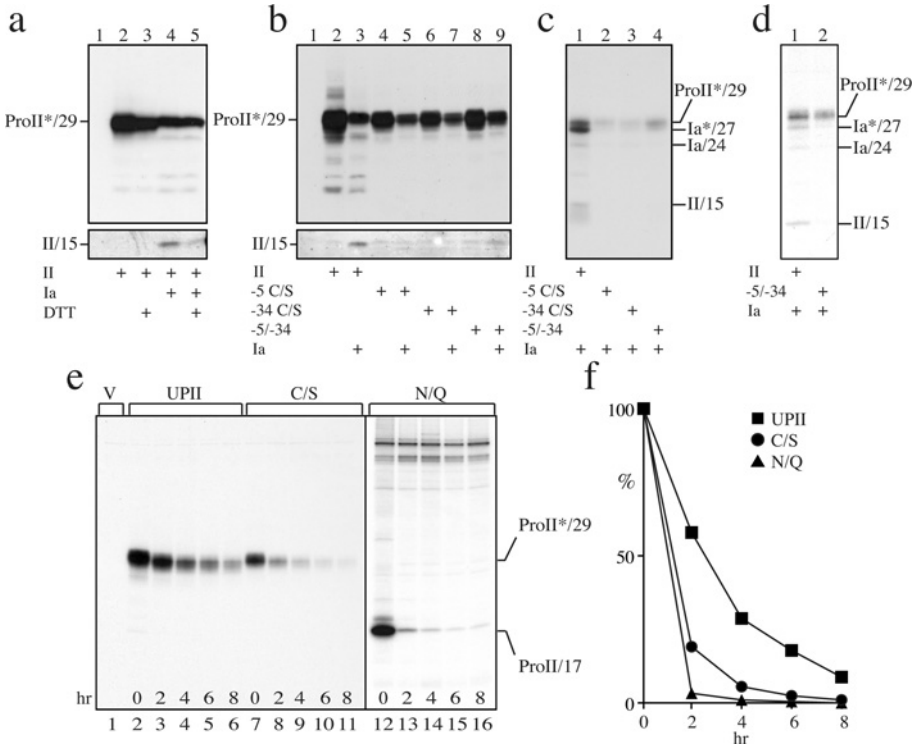

Figure 3 Loss of disulfide linkage in the prosequence blocks UPII maturation

(**a**) Effects of DTT on proUPII processing. COS-1 cells were transfected with an empty pcDNA3 plasmid (lane 1), UPII cDNA or UPII plus UPIa cDNAs (as indicated), and cell lysates were immunoblotted using AE31 (upper panel) or an anti-P4 serum (lower panel). Some of the cells (lanes 3 and 5) were incubated with 5 mM DTT in the culture medium before lysis. Note in lane 5 that DTT partially inhibits the maturation of UPII. (**b**) Mutation of the cysteine residues in the prosequence blocks proUPII maturation. COS-1 cells were transfected with the empty pcDNA3 plasmid (lane 1), or cDNAs of UPII or various UPII mutants in which one of the two, or both, cysteine residues were changed to serine (as indicated). Some cells were double-transfected with UPIa (as indicated). Cell lysates were immunoblotted using AE31 (upper panel) or an anti-P4 serum (lower panel). (**c**) Effects of cysteine mutations on the binding of proUPII to UPIa and the processing of proUPII to mature UPII. Similarly transfected COS-1 cells (as indicated) were [^{35}S]methionine-labelled and immunoprecipitated using the AE31 antibody. Note the inefficient binding of proUPII to UPIa* and the lack of production of mature UPII when one or both cysteine residues were mutated. (**d**) Results from an independent experiment are shown to better illustrate the inefficient binding of proUPII to UPIa*. (**e**) Effects of cysteine and N-glycosylation site mutations on the stability of proUPII. COS-1 cells were transfected with the control pcDNA3 plasmid (lane 1), or cDNAs of wild-type UPII (lanes 2–6), UPII double cysteine mutant [Cys(−5/−34)Ser; lanes 7–11] or UPII triple sugar mutant [Asp(−19/−28/−57)Gln; lanes 12–16], radiolabelled for 1 h, and chased for the indicated period. The cell lysates were immunoprecipitated using the AE31 antibody. (**f**) Quantification of the bands shown in (**e**) by densitometry. Results shown in (**a**–**e**) are representative of three independent experiments.

(circled in Figure 1a) from asparagine to glutamine to generate seven UPII sugar mutants [M1: N(−19)Q; M2: N(−28)Q; M3: N(−57)Q; M4: N(−19/−28)Q; M5: N(−28/−57)Q; M6: N(−57/−19)Q; and M7: N(−19/−28/−57)Q] and assessed the conformational changes in these mutants based on their immunoreactivities to all the UPII-related antibodies by immunoprecipitation (Figure 4). Although, with the exception of the triple mutant, the abolishment of N-glycosylation did not alter significantly their AE31 immunoreactivities (Figure 4a), the proUPII mutants became much more immunoreactive to the anti-Pro (Figures 4b and 4c), anti-P2 (Figure 4d), anti-P1 (Figure 4e), anti-P3 (Figure 4f), as well as anti-P4 (Figure 4g) antibodies. These results are reminiscent of those of the cysteine mutants, and suggest that both N-glycosylation and disulfide bond formation are important in maintaining a compact proUPII conformation.

Inhibition of N-glycosylation using tunicamycin blocked completely the processing of proUPII into the mature UPII in the double UPII/UPIa transfectants (Figure 5a, lanes 7, and 8), suggesting that N-glycosylation of proUPII plays a crucial role in UPII maturation. In the same experiment, we chose to treat the cell lysates with DSS and found that proUPII by itself tends to aggregate, while the presence of UPIa blocked such an aggregation (Figure 5a, lanes 2 and 6). The role of N-glycosylation in UPII maturation was further investigated using asparagine-to-glutamine UPII mutants, and similar blockage of UPII processing was observed for some of these mutants (Figure 5b). The results indicate that mutations of the −19 and −57 N-glycosylation sites disrupt UPII maturation more strongly than do those of the −28 site (Figure 5b). ProUPII with all three N-glycosylation sites mutated became extremely unstable [$t_{1/2}$ (half-life) reduced from ∼3.5 to ∼1 h; Figures 3e, lanes 12–16; and Figure 3f], possibly due to ER-associated degradation; however, this mutant could still bind to UPIa (lane 9 in Figures 5c and 5d) and give rise to a small amount of mature UPII (lane 9 in Figures 5c and 5d).

UPs form heterotetramers in bovine urothelium but form only heterodimers in cultured bovine urothelial cells

To determine how the UPs form dimers, tetramers and higher oligomers, we analysed their aggregation states using the gradient BN-PAGE (blue native PAGE). By analysing the dodecylmaltoside-solubilized proteins of purified bovine urothelial plaques using BN-PAGE, we identified four protein complexes harbouring an apparent molecular mass of ∼250, ∼125, ∼75 and ∼40 respectively (upper panel in Figure 6a). Subsequent analyses using two-dimensional SDS/PAGE/immunoblot revealed that three of the four complexes are UP-positive. They are designated as complexes 1, 2 and 3 in Figures 6(a)–6(e), which have a

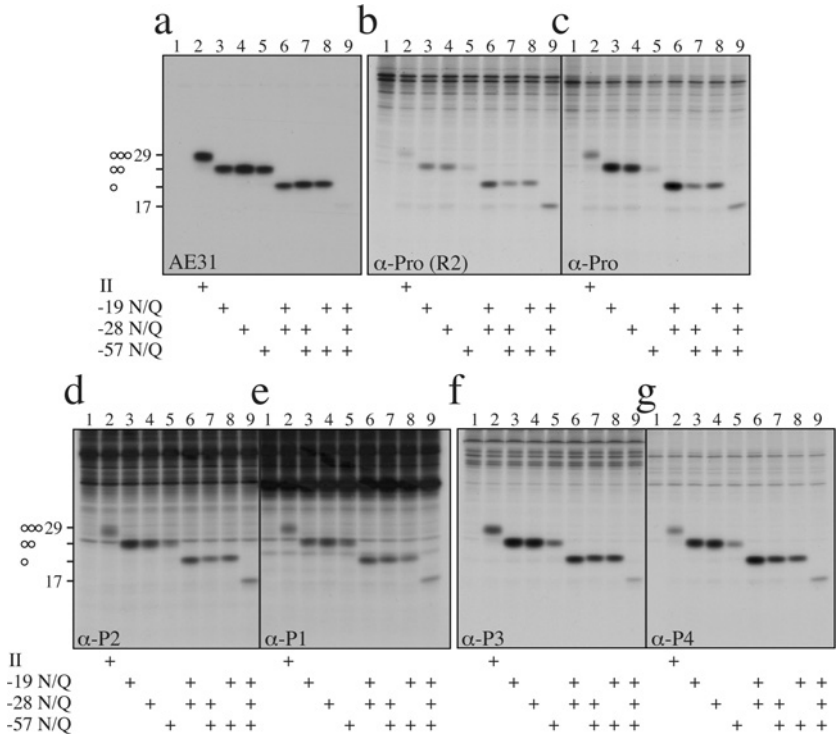

Figure 4 Loss of N-glycosylation leads to major changes in the immunoreactivities of proUPII

COS-1 cells were transfected with the empty pcDNA3 plasmid (lane 1 of each panel), UPII cDNA (lane 2) or various UPII mutants in which one to three of the N-glycosylation sites (positions −19, −28 and −57) were changed to glutamine (as indicated). The cells were labelled with [^{35}S]methionine, and the cell lysates were immunoprecipitated using the AE31 antibody (**a**), two independent rabbit anti-prosequence sera (**b**, **c**) and antibodies to epitopes P2 (**d**), P1 (**e**), P3 (**f**) and P4 (**g**) (also see Figure 1a). Note that the loss of glycosylation did not significantly change the immunoreactivities of the proUPII to the AE31 antibody, but greatly enhanced the immunoreactivities of the proUPII to all other antibodies (except when all three N-glycosylation sites were mutated). Results shown in each panel are representative of three independent experiments.

molecular mass of ∼250, ∼125 and ∼75 kDa respectively. Immunoblot results showed that both complexes 1 and 2 contain UPIa, UPIb, UPII and UPIIIa (Figures 6b–6e) and appear to be, according to their molecular masses, the heterotetramer (complex 2; corresponding to a dumbbell-shaped subunit in a urothelial particle) and the dimeric form of such a heterotetramer (complex 1). Complex 3, containing only UPIb and UPIIIa, corresponds to the UPIb/UPIIIa heterodimer. Similar analyses of the total membrane proteins of cultured bovine urothelial cells revealed the presence of a complex (I) consisting of UPIa/proUPII (or UPIa/UPII) and another (II) containing UPIb/UPIIIa heterodimers, without detectable tetramers (Figures 6g–6j).

Detection of the prosequence in purified urothelial plaques and on the cell surface

Another interesting aspect of the BN-PAGE results is that complex 1 of the urothelial plaque, in addition to UPIa/UPII and UPIb/UPIIIa, also contains proUPII (Figure 6d). This result indicated that both mature UPII and uncleaved proUPII could be a part of the hetero-octamer (Figure 6d), and raised the interesting possibility that the cleaved prosequence might remain attached to the mature UPII in the UP complex even in the mature, surface-expressed urothelial plaques. To test this possibility, we immunoblotted the highly purified, Sarkosyl-washed bovine urothelial plaques using a rabbit antiserum against the UPII prosequence (Figure 7a, lane 2). An intense 24 kDa protein, which was not visible by Coomassie Blue or silver nitrate staining (Figure 7a, lane 1) and thus previously unnoticed, was readily detected. N-terminal sequencing yielded a single sequence of DF(–)ISSLSGLLSPV, thus confirming the prosequence identity of this 24 kDa band. Endo H treatment resulted in a reduction of ∼2 kDa molecular mass (Figure 7a, lane 3), consistent with the fact that the UPII prosequence of *in vivo* urothelium is known to contain a single high-mannose glycan and two complex glycans [28]. Endo F treatment led to an abolition of the band detected with the prosequence antibody, suggesting that the total removal of glycans leads to altered protein conformation and thus abolishes immunoreactivity.

We have shown previously that the AE31 antibody decorates the entire apical surface of bovine urothelial umbrella cells [4,31,38]. This result strongly suggests that the prosequence of UPII remains attached to the UP complexes in mature urothelial plaques. To test this possibility, we stained non-permeabilized COS-1 cells expressing proUPII plus its partner UPIa. Control experiments showed that intact COS-1 cells expressing UPII alone were AE31-negative (Figure 7b), consistent with our previous suggestion that UPII must form a heterodimer with UPIa in order to exit from the ER [28,29]. However, intact COS-1 cells co-expressing both proUPII and UPIa were strongly surface-stained by AE31 (Figure 7c). Intact cultured bovine urothelial cells were also mostly AE31-positive (Figure 7d), suggesting that the AE31 antigen, which is a composite epitope consisting of one segment of the prosequence and another of the mature UPII [28], must be present on the apical surface of these cultured urothelial cells. Interestingly, these AE31-positive cells were UPII- and Ia-negative, whereas those few AE31-negative cells were UPII- and UPIa-positive (Figures 7d and 7e).

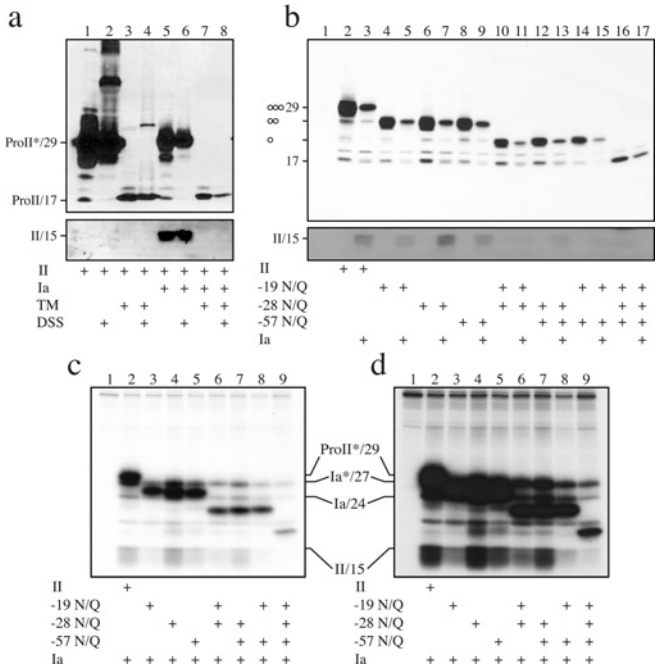

Figure 5 Loss of N-glycosylation of proUPII leads to reduced UPII maturation

(**a**) Effects of tunicamycin on proUPII processing. COS-1 cells were transfected with cDNAs encoding UPII and UPIa (as indicated). Some of the cells were treated with tunicamycin (TM) throughout the whole transfection period before lysis; some of the lysates were subsequently cross-linked using DSS. Cell lysates were then immunoblotted using AE31 (upper panel) or the anti-P4 antibody (lower panel). Note the blockage of proUPII processing by tunicamycin. (**b**) Effect of mutations in the N-glycosylation sites of the prosequence on proUPII processing. COS-1 cells were transfected with the empty pcDNA3 plasmid (lane 1), control UPII cDNA (lanes 2 and 3), or various UPII mutants whose three N-glycosylation sites were mutated in different combinations; some cells were also double-transfected with UPIa (as indicated). The cell lysates were immunoblotted with AE31 antibody (upper panel) or the anti-P4 antibody (lower panel). Note that the mutation of each N-glycosylation site led to a similar size reduction. (**c**) Effects of mutations in the N-glycosylation sites on the binding of proUPII to UPIa. COS-1 cells were transfected with an empty pcDNA3 plasmid (lane 1), UPII and UPIa cDNAs (lane 2) or cDNAs of various UPII sugar mutants plus UPIa (lanes 3-9). The cells were [^{35}S]methionine-labelled, and immunoprecipitated using AE31. (**d**) A longer-exposed version of the gel in (**c**). Note that ablation of N-glycosylation of proUPII does not block the binding of proUPII to UPIa, and that proUPII with mutations in all three N-glycosylation sites can still be processed into mature UPII. Results shown in each panel are representative of three independent experiments.

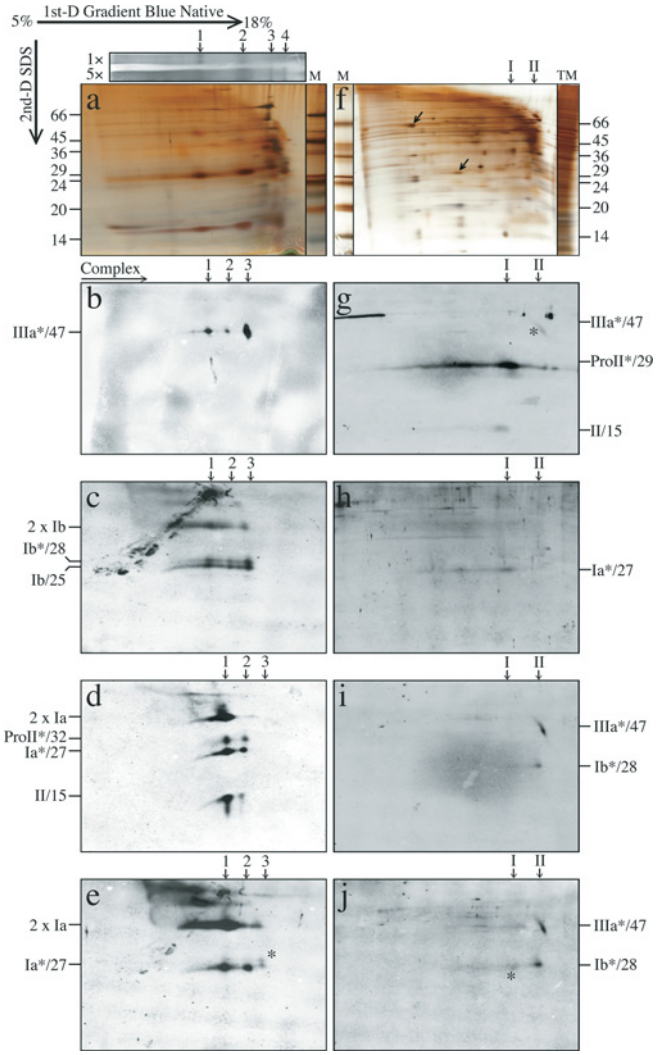

Figure 6 UPs form heterotetramers *in vivo* but only form heterodimers in cultured bovine urothelium

(**a**) Bovine urothelial plaque proteins solubilized in 1.5 % dodecylmaltoside were analysed on a blue native gradient gel (upper panel) followed by SDS/PAGE (silver nitrate staining). M: protein markers. '5 × ' indicates that five times more proteins were loaded than in the lane marked '1 × '. The resolved proteins were transferred to a nitrocellulose membrane and immunoblotted using the (**b**) anti-UPIIIa (AU1) antibody; (**c**) anti-UPIb antibody; (**d**) anti-UPII (P4) plus anti-UPIa plus AE31 antibodies; and (**e**) anti-UPIb plus anti-UPIa antibodies. Note complexes 1 (all four UPs twice), 2 (all four UPs once) and 3 (UPIb and UPIIIa). Also note the presence of proUPII in the heterotetramers, suggesting that the prosequence of UPII is not yet cleaved in some of the heterotetramers. The asterisk in (**e**) marks the UPIb signal. 2 × Ia and 2 × Ib indicate homodimers of UPIa and UPIb respectively; such homodimers often form when UPIa and UPIb in urothelial plaques are analysed by SDS/PAGE. (**f**) Separation of the total membrane proteins of cultured bovine urothelial cells by two-dimensional gel electrophoresis (gradient BN-PAGE followed by SDS/PAGE; silver nitrate staining). The diagonal arrows indicate two non-UP-containing complexes. Abbreviations: M, protein markers; TM, total membranes of cultured bovine urothelial cells. Immunoblot analysis using the (**g**) anti-UPII (P4) plus AE31 plus anti-UPIIIa (AU1) antibodies; (**h**) anti-UPIa antibody; (**i**) anti-UPIb plus anti-UPIIIa (AU1) antibodies; and (**j**) anti-UPIa plus anti-UPIb plus UPIIIa (AU1) antibodies. The asterisks mark the UPIIIa and UPIa signal in (**g**) and (**j**) respectively. Note the heterodimeric UPIa/proUPII (and UPIa/UPII) (I) and UPIb/UPIIIa (II) complexes. Results shown in each panel are representative of two independent experiments.

DISCUSSION

The prosequence of UPII modulates the proper conformation, stability and maturation of proUPII

We have shown previously that the AE31 monoclonal antibody recognizes a composite epitope consisting of two domains, one located near the N-terminus of the prosequence in proUPII (positions −50 to −40) with the other in the middle of the mature sequence (positions +40 to +60) [28]. This result indicates that the AE31-reactive proUPII must assume a hairpin-like structure (Figure 8a; [28]). Our current results indicate that the structure of proUPII is highly governed by the disulfide linkage and N-glycosylation of the prosequence, since abolishing these post-translational modifications using either chemical reagents (2-mercaptoethanol, DTT or tunicamycin) or site-directed mutagenesis leads to significant changes in the proUPII conformation (Figures 2–5 and 8c). Our results indicate that the proUPII was recognized only weakly by antibodies raised against the prosequence ('anti-Pro') or against the mature UPII (anti-UPII) (Figures 1b, 1c and 8a). However, after ablation of the disulfide linkage, proUPII adopted an altered conformation, as indicated by its decreased immunoreactivity to the AE31 antibody and, concomitantly, its greatly increased immunoreactivity to the anti-Pro and anti-UPII antibodies (Figures 2a and 8c). These

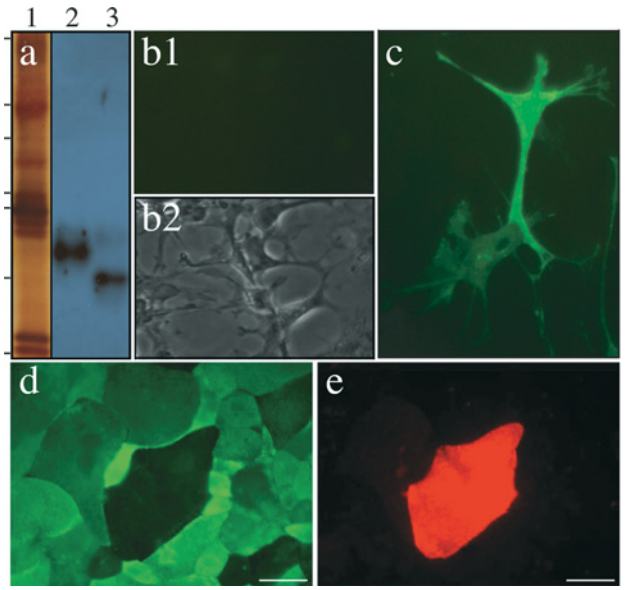

Figure 7 Detection of intact proUPII/pro-UPII on the surface of UPII/UPIa transfectants and cultured bovine urothelial cells

(**a**) SDS/PAGE of bovine urothelial plaque proteins, stained by silver nitrate (lane 1) or immunoblotted using the anti-prosequence antibody (lanes 2 and 3). Lysates in lane 3 were treated with endo H. Molecular mass markers (positions indicated on the left from bottom to top respectively) are 14, 20, 24, 29, 36, 45 and 66 kDa. (**b1**, **b2**) The immunofluorescent and phase-contrast pictures respectively of the non-permeabilized, UPII-transfected COS-1 cells, showing no surface staining by the mouse monoclonal AE31 antibody. (**c**) The non-permeabilized UPII/UPIa-double-transfected COS-1 cells were surface-stained by AE31. The non-permeabilized cultured bovine urothelial cells were double-stained by AE31 (**d**) and the rabbit anti-UPIa antibody (**e**). Note the mutually exclusive staining pattern. Scale bar, 30 μm. Results in each panel are representative of at least three independent experiments.

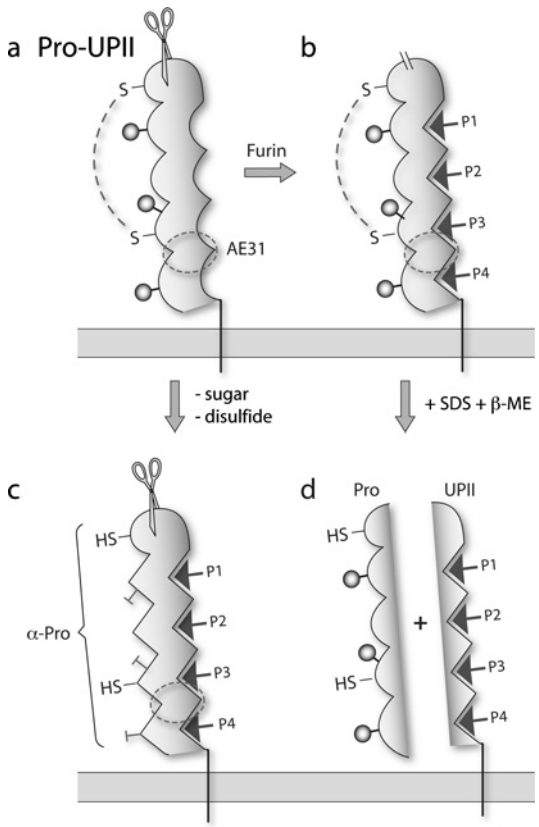

Figure 8 A model of UPII processing and maturation

A schematic model showing (**a**) an intact proUPII consisting of a prosequence (to the left of the furin-cleavage site marked by the scissors) and mature UPII anchored to the lipid bilayer via its C-terminal transmembrane domain. The S–S, small circles and dashed circle denote the disulfide bond, N-glycans and the AE31 epitope respectively. The TGN-associated furin introduces a nick (shown as a break in **b**). Such a complex, in which the cleaved prosequence remains attached to the mature UPII, most probably exists in post-Golgi UP-delivering vesicles and in mature cell-surface-associated urothelial plaques. We hypothesize that this cleavage induces conformational changes in the mature UPII (note shape change of the P1–P4 epitopes from semicircles to zigzags). Similar conformational changes in the mature sequence of proUPII can be induced by a blockage of N-glycosylation and disulfide formation (**c**). The altered conformation of mature UPII persists in SDS/PAGE (**d**).

results indicate that the prevention of disulfide bond formation can lead to a significant change in proUPII conformation even in sequences that are far away from the two cysteine residues (Figure 8c). Moreover, proUPII lacking the disulfide linkage becomes less stable (Figures 3e and 3f), and can hardly interact with UPIa (Figures 3c and 3d); the major structural alteration of the cysteine-mutated proUPII can thus explain the failure of the mutant proUPII to interact with its partner UPIa, to leave the ER, and to give rise to the mature UPII. Related results indicate that the proper glycosylation of the prosequence of proUPII is also important in maintaining the correct conformation of proUPII [28]. Thus, when proUPII is incapable of undergoing N-glycosylation, it folds into a conformation that is, similarly to the cysteine mutants, more readily recognized by the anti-Pro and anti-UPII antibodies, including the distant P4 epitope (Figures 4 and 8c). Functionally, proUPII without N-glycosylation becomes extremely unstable and highly susceptible to degradation (Figures 3e and 3f), and this results in a lower level of mature UPII production (Figure 5b) even though such mutant proUPII can still bind to its partner UPIa (Figures 5c and 5d).

The furin-cleavage site requires a signature sequence: Arg^{-4}-Xaa^{-3}-$(Lys/Arg)^{-2}$-Arg^{-1}; in general, the -1 position must be arginine, and two out of the three residues at -2, -4 and -6 positions must be arginine or lysine for efficient furin cleavage [39]. Although the furin site of proUPII (RGRR) fulfils this requirement, it is unusual that one of the two cysteine residues known to be involved in disulfide bond formation actually resides in the -5 position within the furin-cleavage motif. Our results clearly indicate that this disulfide linkage is required for the furin-mediated processing of proUPII into mature UPII (Figures 3a and 3b).

Taken together, our results indicate that the prosequence requires proper disulfide linkage and N-glycosylation in order to maintain a certain, SDS-resistant, hairpin-like proUPII conformation (Figure 8a) that can bind to UPIa, leading to the formation of the proUPII/UPIa complex that can then exit from the ER (Figure 9). There is previous evidence for membrane proteins adopting a particular conformation or aggregation state that is SDS-resistant. For example, the single-transmembrane-domain glycophorin A can form SDS-resistant homodimers via intermolecular, helix–helix interactions facilitated by the intimate contact of the glycine residues in the transmembrane domains [40–43]. The hairpin conformation of proUPII seems to be unique, however, in that it involves intramolecular, SDS-resistant interactions within an integral membrane protein. Our present study also illustrates the usefulness of using conformation-dependent antibodies to study the conformational changes of membrane proteins during assembly.

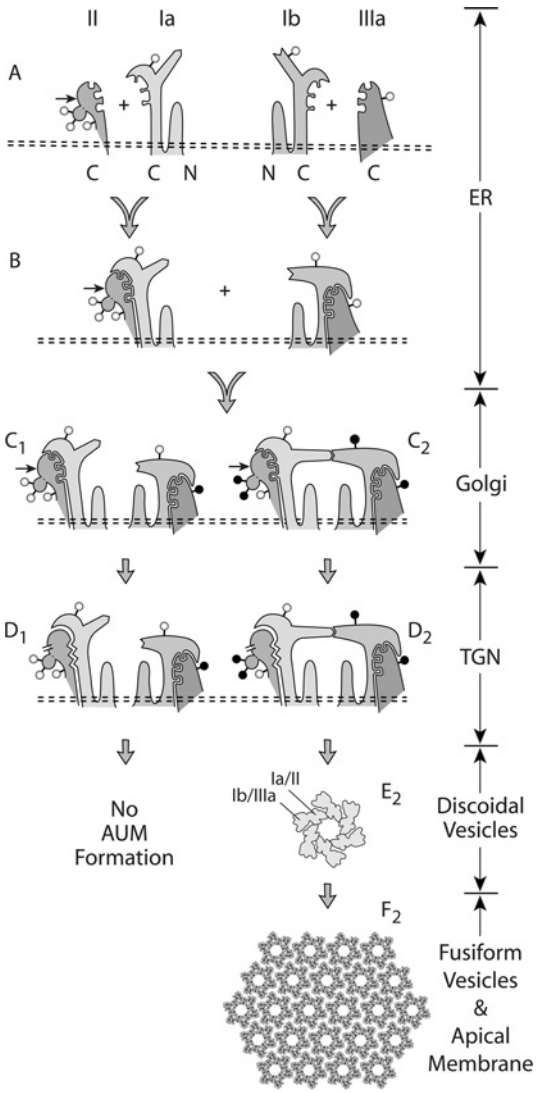

Figure 9 A model of urothelial plaque assembly

This diagram is similar to that presented in our earlier paper [28], except that the newly discovered disulfide bond is incorporated into the prosequence of UPII and that the cleaved prosequence of UPII remains attached to the mature protein even in assembled urothelial plaques (AUM).

UP tetramers are present in purified urothelial plaques but not in cultured bovine urothelial cells

By studying the dodecylmaltoside-solubilized total membranes of the cultured bovine urothelial cells using BN-PAGE, we found that the 29 kDa proUPII of the cultured bovine urothelial cells can interact with UPIa to form only a heterodimer (Figures 6f–6h). Similarly, UPIIIa can interact with UPIb to form another heterodimer (Figures 6f, 6g, 6i and 6j). However, in cultured bovine urothelial cells, there is no formation of UP heterotetramers (Figures 6f–6j); this provides an explanation for the inability of cultured urothelial cells to form the 16-nm urothelial particles. On the other hand, the dodecylmaltoside-solubilized mature urothelial plaques (AUM) contain at least three UP complexes, i.e. a dimer (UPIb/UPIIIa; complex 3), a tetramer (1 × UPIa/32 kDa proUPII/UPIb/UPIIIa; complex 2) and a higher oligomer containing a dimer of the tetramers (2 × UPIa/32 kDa proUPII/UPIb/UPIIIa; complex 1) (Figures 6a–6e). It is known that while the prosequence of the 29 kDa proUPII contains three high-mannose glycans, the prosequence of the 32 kDa proUPII has two of its three high-mannose glycans processed into complex glycans [28]. Therefore the association of the 32 kDa proUPII with UP heterotetramers (UPIa/32 kDa proUPII/UPIb/UPIIIa) supports the possibility that the complex glycan modification of the proUPII is necessary for the formation of UP heterotetramers [28].

In the studies on the oligomerization status of UPs in cultured urothelial cells and urothelial plaques (AUM) using the blue native gel, we detected uncleaved proUPII co-migrating with the mature UPIa/UPII complexes (Figures 6d, 6e, 6g and 6h). We previously assumed that the cleavage of the prosequence of proUPII by furin results in the removal and possible degradation of the prosequence. Our current results indicate, however, that at least some of the uncleaved 32 kDa proUPII remains associated with the purified urothelial plaques (Figure 6d). It is possible that the heterotetramer-associated 32 kDa proUPII comes from the fraction of UP complexes in the early endomembranes (such as the Golgi apparatus) and immature vesicles, which are co-purified with the cell surface-associated, mature urothelial plaques of umbrella cells. Nevertheless, our data raise the possibility that this 32 kDa proUPII is actually assembled into the mature urothelial plaques as a major UPII component, that its prosequence is cleaved by furin either in the mature fusiform vesicles *en route* to the cell surface or on the cell surface itself [44] and that the cleaved prosequence remains associated with the 'mature UPII' sequence in the urothelial plaques (see below).

The prosequence of UPII is cleaved but is still associated with mature UPII in the urothelial particles

Our BN-PAGE data showed that UPIa was consistently associated with UPII and proUPII in the same heterodimeric and heterotetrameric complexes (Figures 6d, 6e, 6g and 6h). These data indicate that the nicked pro-UPII (i.e. the 15 kDa mature UPII with its associated prosequence) can be assembled into the urothelial particles that are present on the urothelial apical surface (Figure 9) and that the 15 kDa UPII is derived from the nicked pro-UPII only during SDS/PAGE (Figure 8d). Such a hypothesis provides an explanation for many otherwise perplexing observations. (i) The AE31 antibody (which only recognizes proUPII [28]) can immunoprecipitate the mature 15 kDa UPII (lane 3 in Figure 1b; lane 2 in Figure 1d; lane 1 in Figures 3c and 3d; lanes 2–9 in Figures 5c and 5d; [4]). This can be explained if the 15 kDa is originally pulled down as the nicked pro-UPII (still recognized by the AE31 antibody) and is later dissociated from its prosequence during SDS/PAGE. (ii) The AE31 antibody can stain the apical surface of umbrella cells in normal urothelium, and the surface of cultured urothelial cells and UPII/UPIa-double-transfected COS-1 cells ([4]; Figures 7c and 7d, and results not shown). This can now be explained by the fact that the AE31 antibody can also recognize the nicked pro-UPII.

Taken together, our studies suggest that the prosequence of UPII plays an important role in regulating the processing of UPs and the differentiation-dependent formation of AUM. Our model of urothelial plaque assembly (Figure 9) provides an improved understanding of the roles of a variety of factors affecting the oligomerization of integral membrane proteins, and is well suited to further studies to elucidate the mechanisms and signalling that regulate such complex processes.

We thank Herbert Samuels, Richard Margolis, Eugene Marcantonio and Liyu Tu for useful discussions; Xiangpeng Kong, Angel Pellicer and Xue-Ru Wu for a critical reading of this paper; Gordon Cook for illustrations; and Herbert Lepor and the late Irwin Freedberg for

their support. The present studies were supported by NIH (National Institutes of Health) grants DK39753, DK52206 and P30 CA16087.

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Received 11 March 2008/9 May 2008; accepted 16 May 2008
Published as BJ Immediate Publication 16 May 2008, doi:10.1042/BJ20080550