MAL facilitates the incorporation of exocytic uroplakin-delivering vesicles into the apical membrane of urothelial umbrella cells

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ABSTRACT The apical surface of mammalian bladder urothelium is covered by large (500–1000 nm) two-dimensional (2D) crystals of hexagonally packed 16-nm uroplakin particles (urothelial plaques), which play a role in permeability barrier function and uropathogenic bacterial binding. How the uroplakin proteins are delivered to the luminal surface is unknown. We show here that myelin-and-lymphocyte protein (MAL), a 17-kDa tetraspan protein suggested to be important for the apical sorting of membrane proteins, is coexpressed with uroplakins in differentiated urothelial cell layers. MAL depletion in Madin–Darby canine kidney cells did not affect, however, the apical sorting of uroplakins, but it decreased the rate by which uroplakins were inserted into the apical surface. Moreover, MAL knockout in vivo led to the accumulation of fusiform vesicles in mouse urothelial superficial umbrella cells, whereas MAL transgenic overexpression in vivo led to enhanced exocytosis and compensatory endocytosis, resulting in the accumulation of the uroplakin-degrading multivesicular bodies. Finally, although MAL and uroplakins cofloat in detergent-resistant raft fractions, they are associated with distinct plaque and hinge membrane subdomains, respectively. These data suggest a model in which 1) MAL does not play a role in the apical sorting of uroplakins; 2) the propensity of uroplakins to polymerize forming 16-nm particles and later large 2D crystals that behave as detergent-resistant (giant) rafts may drive their apical targeting; 3) the exclusion of MAL from the expanding 2D crystals of uroplakins explains the selective association of MAL with the hinge areas in the uroplakin-delivering fusiform vesicles, as well as at the apical surface; and 4) the hinge-associated MAL may play a role in facilitating the incorporation of the exocytic uroplakin vesicles into the corresponding hinge areas of the urothelial apical surface.

INTRODUCTION

Epithelial cells perform many vectorial functions that require the polarized distribution of membrane proteins into apical and basolateral compartments, a process that has been studied extensively. Apical targeting is a two-step process: a membrane protein is first sorted at the trans-Golgi network (TGN) level into apical surface–destined vesicles, which are subsequently incorporated, in a regulated manner in the case of storage vesicles, into the apical surface. Very few proteins are known to play a role in directing the apical targeting of membrane proteins (Rodriguez-Boulan et al., 2005; Mellman and Nelson, 2008; Folsch et al., 2009; Carmosino et al., 2010b). One of them is myelin-and-lymphocyte protein (MAL;
VIP17), a 17-kDa integral membrane protein with four transmembrane domains expressed in T lymphocytes, myelin-forming cells, and many polarized epithelial cells (Alonso and Weissman, 1987; Schaeren-Wiemers et al., 1995; Zacchetti et al., 1995; Liebert et al., 1997; Frank et al., 1998; Cheong et al., 1999; Marazuela et al., 2003). Based largely on the in vitro data from Madin–Darby canine kidney (MDCK) cells, it has been suggested that this protein plays a key role in regulating membrane proteins to the apical surface of polarized epithelial cells (Puertollano et al., 1999; Martin-Belmonte et al., 2000; Rodriguez-Boulan et al., 2005).

Mammalian bladder urothelium consists of basal, intermediate, and terminally differentiated, superficial umbrella cells (Khandelwal et al., 2009; Wu et al., 2009). The luminal membrane of the umbrella cells is almost completely covered by two-dimensional crystals of hexagonally packed 16-nm particles (urothelial plaques; Hicks and Ketterer, 1969; Vergara et al., 1969; Kachar et al., 1999; Min et al., 2006) comprising four major integral membrane proteins—the uroplakins (UPs) Ia (26 kDa), Ib (27 kDa), II (15 kDa), and IIIa (47 kDa; Wu et al., 2006) comprising four major integral membrane proteins. The crystalline nature of the urothelial plaques, which can be readily purified in milligram quantities (Wu et al., 1999; Liang et al., 2001; Min et al., 2003), allows for their detailed structural analysis (Hicks and Ketterer, 1969; Vergara et al., 1969; Kachar et al., 1999; Min et al., 2006). Such studies showed that the UPIa/I and UPIb/III heterodimers are associated with the inner and outer subdomains of the 16-nm particle, respectively (Min et al., 2003). Finally, the urothelial apical surface, which is accessible via a catheter, provides one of the most impressive permeability barriers known to exist in nature (Negrete et al., 1996; Hu et al., 2000, 2002; Kong et al., 2004), and one of the uroplakins, UPIa, serves as the receptor for the type 1 fimbriated Escherichia coli that cause >85% of all the cases of urinary tract infections (Wu et al., 1996; Zhou et al., 2001; Wang et al., 2009). Therefore mammalian bladder urothelium constitutes a physiologically relevant system that provides excellent opportunities for studying mechanisms of membrane trafficking.

In this work, we investigated the role of MAL in uroplakin transport, using knockout mice lacking MAL or uroplakins, transgenic mice overexpressing MAL, and MDCK cells in which MAL expression was knocked down. Our data allowed us to dissect the apical targeting of uroplakins into two phases. We conclude that MAL does not play a role in the apical sorting of uroplakins; instead, it plays an important role in facilitating the subsequent incorporation of the uroplakin vesicles into the urothelial apical membrane. Given the widespread distribution of MAL in epithelia, it is possible that these conclusions are applicable to other specialized epithelial cell types and tissues.

RESULTS

Uroplakins are targeted to the apical surface of urothelial umbrella cells

Although we showed previously that uroplakins are the major constituents of the apical surface of the umbrella cell (Wu et al., 1990; Wu and Sun, 1993; Lin et al., 1994; Yu et al., 1994), it is unclear to what extent uroplakins are selectively targeted to the urothelial apical surface. We showed that urothelial plaques purified from mouse urothelium (Figure 1A) using sucrose density gradient centrifugation coupled with Sarkosyl wash contained four major uroplakins—uroplakins Ia, Ib, II, and IIIa (marked by filled dots in Figure 1B, lane 2; Zhou et al., 2001)—plus an unknown ~18-kDa protein (Figure 1B, lane 2; open dot). In vivo biotinylation of intact mouse urothelial apical surface (Figure 1C and Supplemental Figure 1, A and B) resulted in the strong labeling of uroplakins Ia, Ib, and IIIa and weak labeling of UPIII (Figure 1D), which together accounted for >80% of the total biotin label. Pretreatment of mouse urothelium with 0.1% Triton X-100 led to massive nonspecific biotinylation of cytoplasmic proteins (Supplemental Figure 1C and unpublished data), thus establishing the specificity of the surface labeling. Immuno–electron microscopy (immuno-EM) localization showed that uroplakins are exclusively associated with the luminal surface of the mouse urothelial umbrella cells, with negligible basolateral labeling (Figure 1E and inset), thus establishing that uroplakins are selectively targeted to the apical surface of mouse bladder urothelium. These data also confirmed that certain domains of the uroplakin proteins are exposed on the luminal side of the apical surface (Yu et al., 1994).

MAL and uroplakin are coexpressed in upper urothelial cells

We identified the electrophoretically purified 18-kDa protein (Figure 1B, lane 7) by N-terminal sequencing as MAL (Alonso and Weissman, 1987; Schaeren-Wiemers et al., 1995), a result confirmed by immunoblotting using a goat antiserum to MAL (Figure 1B, lane 9). Mouse urothelium is a stratified epithelium consisting of a basal layer of relatively undifferentiated germinative cells, one or two layers of intermediate cells, and a top layer of terminally differentiated umbrella cells (Hicks, 1975; Romih et al., 2005; Khandelwal et al., 2009). Immunofluorescence staining of mouse bladder sections showed that MAL and uroplakin IIIa largely coexpressed in the intermediate and umbrella cell layers (Figure 2, A and B), consistent with an earlier observation that MAL is associated with urothelial differentiation in vitro (Liebert et al., 1997). High-resolution confocal microscopic revealed, however, that MAL expression preceded that of uroplakins during urothelial differentiation, as only the former was present in the apical portion of the basal cells (white downward arrows in Figure 2B). On the other hand, the apical surface of the umbrella cells, which was clearly uroplakin positive, was weakly stained by antibody to MAL (Figure 2B, 3, small arrows). Finally, confocal microscopy showed that MAL staining frequently flanked the uroplakin-positive vesicles (Figure 2, C–E).

MAL knockout in MDCK cells has no effect on the transport of uroplakins to the apical surface but diminishes their rate of apical incorporation

To study the mechanism by which uroplakins are apically targeted, we expressed uroplakins in polarized MDCK cells. We found that singly expressed uroplakins (with the exception of UPIb) remained cytoplasmic and failed to reach the cell surface. These results are consistent with our earlier results obtained with nonpolarized 293T cells (Tu et al., 2002; Hu et al., 2005) and suggest that these singly expressed uroplakins were trapped in the endoplasmic reticulum (ER; Figure 3A). However, coexpression of uroplakins UPIa and Ib with UPIII and IIIa, respectively, in MDCK cells allowed them to exit as UPIa/I and UPIb/III heterodimers from the ER and to reach the apical cell surface (Figure 3B), colocalizing with MAL, which is known to be associated with the apical membrane (Supplemental Figure S2; Frank et al., 1998; Cheong et al., 1999; Puertollano and Alonso, 1999). That these uroplakin pairs became exposed on the apical surface is supported by the observations that they could be apically biotinylated (Figure 3E) and detected immunologically in intact MDCK cells (unpublished data; also see Thumbikat et al., 2009). These results showed that uroplakin Ia/I and Ib/IIIa heterodimers, and even the singly expressed UPIb, harbored signals allowing them to be transported to the apical plasma membrane domain in a
the MDCK monolayer as measured by transepithelial resistance was
Supplemental Figure 3A). Our data indicated that the integrity of
apical surface by treating the cells with small interfering RNA (siRNA;  MDCK cells (Puertollano et al., 1999), in uroplakin delivery to the
apical surface (arrow), fusiform vesicles (asterisks), and multivesicular bodies (#). (B) Identification of MAL. Lane 1, molecular weight markers. Proteins of purified mouse urothelial plaques were resolved by SDS–PAGE and stained by Coomassie brilliant blue (lane 2). The four filled dots (from the top down) denote the 47-kDa UPIIIa, 28-kDa UPIb, 27-kDa UPla, and 15-kDa UPII, which were identified by immunoblotting using monospecific antibodies (lanes 3–6). The open dot denotes an ~18-kDa unknown protein later identified as MAL. Lane 7, electrophoretically purified 18-kDa band identified by N-terminal sequencing as MAL (see the text). Lane 8, electrophoretically purified 15-kDa UPII. Lanes 9 and 10, immunoblotting of samples shown in lanes 7 and 8 using a goat antibody to MAL. (C) Biotinylation of the mouse urothelial apical surface. Biotin was visualized using FITC-conjugated streptavidin (green fluorescence; nuclei counterstained in red). (D) The surface-biotinylated mouse bladder urothelial apical proteins are predominately uroplakins. Greater than 80% of all the biotinylated surface proteins are in the gradient-purified and Sarkosyl-insoluble uroplakin fraction. Lane 1, intact biotinylated membrane proteins, which were additionally treated with (2) Endo H and (3) Endo F. Because each uroplakin exhibits characteristic size reduction after Endo H and F treatment, these treatments, coupled with immunoblotting using monospecific antibodies to uroplakins, provide unambiguous identification of individual uroplakins. Lanes 4–15, the same three samples immunoblotted using antibodies to uroplakins la (lanes 4–6), UPlb (lanes 7–9), UPII (lanes 10–12), and UPIIIa (lanes 13–15). The four filled dots next to lane 1 denote, from top, UPIIIa, Ib, Ia, and II. Note that all the major biotinylated bands can be accounted for by uroplakins (Ia, Ib, and IIIa strongly and UPII weakly). (E) Immuno-EM localization of uroplakins in two neighboring (polarized) urothelial umbrella cells showing tight junction (TJ), fusiform vesicles (asterisk), apical (black downward arrow) and lateral membrane (open arrow in main panel and black arrows in inset), and lumen (L). Note the exclusive association of uroplakin-immunogold particles with the apical surface (black arrow in main panel), with none associated with the basolateral surface (open arrow). Scale bars, 1 µm (A, E) and 50 µm (C).

![Diagram](image-url)

**FIGURE 1:** Uroplakins are apically targeted. (A) Transmission electron microscopy of a mouse urothelial umbrella cell showing the rigid-looking plaques covering the apical surface (arrow), uroplakin pairs UPIIa/I and UPIIb/IIIa (Figure 3F). To assess the role of MAL in regulating the rate of uroplakin apical incorporation, we blocked the apically exposed amino groups using sulfo-succinimidyl 3-(4-hydroxyphenyl) propionate (sulfo-SHPP), followed by biotinylating the newly exposed apical proteins either immediately (Figure 3C, lanes 1 and 2) or after 6 h (Figure 3C, lanes 3 and 4). Consistent with earlier data (Puertollano et al., 1999), MAL knockdown resulted in a significant reduction in the incorporation of proteins (per 6 h) into the apical membrane. These include the endogenous proteins (~60% decrease; Figure 3C, lanes 3 and 4), as well as exogenous, transfected proteins, including hemagglutinin (Figure 3D, lanes 3 and 4; 20–30% decrease). Similar results were obtained with transfected uroplakin Ib/IIa pair (Figure 3E, lanes 3 and 4; 60–70% reduction). These results were reproduced in three independent experiments. Given that MAL knockdown was previously shown to selectively affect MAL levels but left unaltered the content of other endogenous or exogenous proteins analyzed (Cheong et al., 1999; Puertollano et al., 1999; Martin-Belmonte et al., 2000, 2001), it is unlikely that the observed alterations were due to changes in the total content of hemagglutinin or UPIIa. Taken together, these data indicated that MAL knockdown had no effect on the total content of apical uroplakins (Figure 3F) but significantly reduced the rate of uroplakin incorporation into the apical membrane (Figure 3E).

**Deficiency or overexpression of MAL in vivo has no effect on the apical association of uroplakins**

To study the functional roles of MAL in vivo in uroplakin trafficking, we studied uroplakin delivery to the umbrella cell plasma membrane in MAL-knockout mice (Scharen-Wiemers et al., 2004), whose urothelium, as expected, had no detectable MAL message or protein (Figure 4, A–C). Consistent with the results in MAL-knockdown MDCK cells (Figure 3F), the apical distribution of uroplakins was unaffected (Figure 4, D–F). Parallel analyses of the MAL-overexpressing mouse urothelium (Frank et al., 2000) indicated that it had an elevated (threefold to fivefold) level of MAL message and protein (Figure 4, A–C). Immuno-EM localization showed, again, the exclusive association of nonurothelial cell type, indicating that no urothelium-specific factors are required for this process.

We studied the role of MAL, which is present endogenously in MDCK cells (Puertollano et al., 1999), in uroplakin delivery to the apical surface by treating the cells with small interfering RNA (siRNA; Supplemental Figure 3A). Our data indicated that the integrity of the MDCK monolayer as measured by transepithelial resistance was unaffected by the siRNA treatment (Supplemental Figure 3B) and that the siRNA-treated MDCK cells had no detectable MAL (Figure 3, C–E). Confocal microscopy showed that MAL knockdown had no effects on the basolateral distribution of the endogenous E-cadherin or the normal apical expression of the endogenous gp135 and gp114 (Figure 3F). It also had no effects on the apical delivery of the transfected uroplakin pairs UPIIa/I and UPIIb/IIIa (Figure 3F). To assess the role of MAL in regulating the rate of uroplakin apical incorporation, we blocked the apically exposed amino groups using sulfo-succinimidyl 3-(4-hydroxyphenyl) propionate (sulfo-SHPP), followed by biotinylating the newly exposed apical proteins either immediately (Figure 3C, lanes 1 and 2) or after 6 h (Figure 3C, lanes 3 and 4). Consistent with earlier data (Puertollano et al., 1999), MAL knockdown resulted in a significant reduction in the incorporation of proteins (per 6 h) into the apical membrane. These include the endogenous proteins (~60% decrease; Figure 3C, lanes 3 and 4), as well as exogenous, transfected proteins, including hemagglutinin (Figure 3D, lanes 3 and 4; 20–30% decrease). Similar results were obtained with transfected uroplakin Ib/IIa pair (Figure 3E, lanes 3 and 4; 60–70% reduction). These results were reproduced in three independent experiments. Given that MAL knockdown was previously shown to selectively affect MAL levels but left unaltered the content of other endogenous or exogenous proteins analyzed (Cheong et al., 1999; Puertollano et al., 1999; Martin-Belmonte et al., 2000, 2001), it is unlikely that the observed alterations were due to changes in the total content of hemagglutinin or UPIIa. Taken together, these data indicated that MAL knockdown had no effect on the total content of apical uroplakins (Figure 3F) but significantly reduced the rate of uroplakin incorporation into the apical membrane (Figure 3E).
MAL facilitates exocytic fusion (which are lined with uroplakin-positive plaques; Figure 5, F–H; and unpublished data). These results are in complete agreement with our MDCK cell data (Figure 3, C and D) and suggest that MAL depletion retards the apical incorporation of uroplakins. These data also indicate that MAL plays an important role in determining the steady-state status of the uroplakins, that is, whether the uroplakins exist mainly in fusiform vesicles (of the exocytic/biogenetic pathway) or the multivesicular vesicles (of the endocytic/degradative pathway).

MAL is associated with the hinge areas of the uroplakin-delivering vesicles

To determine the spatial relationship between MAL and uroplakin, we localized MAL and uroplakins in mouse urothelial umbrella cells (Figure 6A) by immuno-EM. Whereas uroplakins were associated with the two plaques in a fusiform vesicle (Figure 4, D and G; Figure 6E; also see Liang et al., 2001), MAL was found to be associated with the uroplakin-free hinge areas (Figure 6, B–E). In the apical surface, which also contained uroplakin plaques interconnected by hinge areas, MAL was again associated with the hinges (Figure 6F). The association of MAL with the hinges of the apical surface could be seen more clearly in the MAL-overexpressing bladder urothelium (Figure 6G). MAL was also detected at the hinges of the multivesicular bodies (unpublished data). These results indicate that even though MAL and uroplakins coexist in the same fusiform vesicles, they are associated with distinct membrane subdomains.

Uroplakin knockout leads to the accumulation of small, MAL-containing vesicles

To assess the effects of removing the uroplakin cargoes from the exocytic fusiform vesicles, we examined the urothelium of uroplakin-knockout mice (Hu et al., 2000; Kong et al., 2004). Electron microscopy showed that uroplakin depletion led to the complete replacement of the fusiform vesicles by numerous rounded vesicles that were 200–300 nm in diameter (Figure 7, A–C; Hu et al., 2002; Kong et al., 2004). Immunofluorescence staining and immuno-EM localization data showed that these uroplakin-depleted vesicles were MAL positive (Figure 7D) and accumulated toward the subapical zone (Figure 7, B, D and E).

MAL and uroplakins are associated with lipid rafts

MAL has been shown to be lipid raft associated in MDCK and Fischer rat thyroid FRT cells (Zacchetti et al., 1995; Martin-Belmonte et al., 1998, 2001), and this has been proposed to be a mechanism for MAL-mediated sorting of proteins to the apical surface of epithelial cells (Cheong et al., 1999; Puertollano et al., 1999; Martin-Belmonte et al., 2000). To see whether MAL and uroplakins are raft associated in mouse urothelial cells, we prepared an extract of the uroplakin-knockout mice.
Figure 3: Depletion of MAL in MDCK cells does not affect the apical sorting of uroplakins, but it reduces their rate of apical incorporation. (A) Individual uroplakins (except UPIb) expressed alone in MDCK cells fail to reach the apical surface. MDCK cells transfected with uroplakin Ia, II, Ib, and IIIa were fixed at 36 h posttransfection, permeabilized with 0.5% Triton X-100 at 25°C for 10 min, double stained with rabbit antibodies to the indicated uroplakins (red) and a rat monoclonal antibody to the ZO1 tight junction apical marker (green), and observed by confocal microscopy. (B) Uroplakins expressed as a UPIa/II or UPIb/IIIa pair (brackets) can reach the apical surface. Top, the localization of ZO1 (green) and E-cadherin (red) as markers for the apical boundary and basolateral membrane. (C) siRNA knockdown of the MAL reduces the delivery rate of apical proteins. MDCK cells were transfected with a control (scrambled; odd-numbered lanes) or a double-stranded siRNA (siRNA200; even numbered) that knocked down the endogenous dog MAL expression (Supplemental Figure S3), seeded into a Transwell, and incubated at 37°C for 36 h. The free amino groups exposed on the apical cell surface were blocked by applying a solution of sulfo-SHPP to the apical compartment of the Transwell, and the newly arrived apical surface proteins were tagged by sulfo-NHS-biotin either immediately (lanes 1 and 2) or after 6 h at 37°C (lanes 3 and 4). Newly exposed apical surface proteins were detected, after SDS-PAGE, using peroxidase-labeled streptavidin (top), and MAL was detected using confocal microscopy. (D) Uroplakins expressed as a UPIa/II or UPIb/IIIa pair (brackets) can reach the apical surface. Top, the localization of ZO1 (green) and E-cadherin (red) as markers for the apical boundary and basolateral membrane. (C) siRNA knockdown of the MAL reduces the delivery rate of apical proteins. MDCK cells were transfected with a control (scrambled; odd-numbered lanes) or a double-stranded siRNA (siRNA200; even numbered) that knocked down the endogenous dog MAL expression (Supplemental Figure S3), seeded into a Transwell, and incubated at 37°C for 36 h. The free amino groups exposed on the apical cell surface were blocked by applying a solution of sulfo-SHPP to the apical compartment of the Transwell, and the newly arrived apical surface proteins were tagged by sulfo-NHS-biotin either immediately (lanes 1 and 2) or after 6 h at 37°C (lanes 3 and 4). Newly exposed apical surface proteins were detected, after SDS-PAGE, using peroxidase-labeled streptavidin (top), and MAL was detected using an anti-MAL antibody (bottom). Note the efficient depletion of MAL expression and the ~60% reduction in the apical delivery of endogenous surface proteins. (D, E) MAL depletion reduces the apical incorporation rate of hemagglutinin (HA) and uroplakins. MDCK cells were transfected with control scrambled siRNA (single-numbered lanes) or siRNA200 RNA (even numbered) and (D) infected with influenza virus or (E) transfected with cDNAs encoding the UPIb/IIIa uroplakin pair. Newly appearing apical surface proteins were detected as described in C either immediately (lanes 1 and 2) or after 6 h (lanes 3 and 4). These biotin-tagged apical proteins were affinity pulled down using streptavidin beads and blotted using antibodies to HA (D) or UPIIa (E). Note that MAL depletion led to ~20–30% decrease in the apical delivery of HA and ~60–70% in that of UPIIa. Similar results were obtained in three independent experiments. (F) MAL depletion had no effect on the apical distribution of uroplakins, gp135, and gp114. MDCK cells were transfected with control siRNA (top, Control) or siRNA200 (bottom, Knockdown) and were transfected 36 h later with cDNAs encoding UPIa/II or UPIb/IIIa. The transfected cells were grown for 36 h, fixed, Triton permeabilized, and immuno-stained for E-cadherin (an endogenous basolateral marker), gp135, and gp114 (both endogenous apical markers), uroplakin II (for the UPIa/II cotransfection), or uroplakin Ii (for UPIb/IIIa) and visualized by confocal microscopy. Top, x-z images of a vertical section. Bottom, x-y plane (projected z-series). Note that MAL depletion had no effect on the apical targeting of uroplakins II and IIIa, gp135, and gp114. Bar, 10 µm.

Discussion
MAL does not play a role in the sorting of uroplakins to the apical surface
Our biotinylation (Figure 1, C and D) and immuno-EM localization data (Figure 1E) established that uroplakins are major integral membrane protein components of the urothelial luminal surface and that uroplakins are apically targeted. Transfection studies of MDCK cells show that the formation of correct uroplakin pairs (UPIa/II and UPIb/IIIa) is a prerequisite for their ER-exit and apical delivery (Figure 3B; with the exception of UPIb, which can exit alone—see Figure 3A). Although these pair-formation data are consistent with our earlier observations made in the nonpolarized 293T cells (Tu et al., 2002; Hu et al., 2005, 2008), the use of polarized MDCK cells here enabled us to demonstrate for the first time that uroplakins can be sorted to the apical membrane in a nonurothelial cell type, thus proving that these proteins must harbor intrinsic signals for apical delivery, without the need for urothelium-specific factors. Finally, we demonstrated that MAL depletion, in MDCK cells (Figure 3) as well as in vivo in the urothelium of the MAL-knockout mice (Figure 4), had no effect on the transport of uroplakins to the apical domain, indicating that MAL is not required for the apical sorting of these membrane proteins.

MAL facilitates the incorporation of the fusiform vesicles into the apical urothelial membrane
Our data indicate that although MAL is dispensable for the sorting of uroplakins into apically destined fusiform vesicles, it plays a role in the incorporation of these exocytic uroplakin-delivering vesicles into the apical surface. First, knockdown of MAL in MDCK cells reduced the rate by which uroplakins (Figure 3E), hemagglutinin (Figure 3D), and...
many other endogenous proteins (Puertollano et al., 1999; Martin-Belmonte et al., 2000; Figure 3C) appeared on the apical surface. Second, ablation of the mouse MAL gene led to the accumulation of fusiform vesicles (Figure 5D) and an increase in the cellular uroplakin content (Figure 4B), suggesting a partial blockage of the incorporation of the uroplakin-delivering vesicles into the luminal surface (Figure 8B). Third, MAL overexpression led to the partial replacement of fusiform vesicles by multivesicular bodies (Figure 5, F–H), suggesting enhanced exocytosis and compensatory endocytosis (Truschel et al., 2002; Gundelfinger et al., 2003; Vogel, 2009; Khandelwal et al., 2010). This interpretation is consistent with the fact that the multivesicular bodies of urothelial umbrella cells are often lined with uroplakin plaques, suggesting their involvement in the recycling (Back et al., 2010) or, more likely, lysosomal degradation of uroplakins (Amano et al., 1991; Khandelwal et al., 2010). Finally, EM localization data showed that MAL could reach the apical surface of MDCK cells (Supplemental Figure S2; Puertollano and Alonso, 1999) and can be recycled to the TGN, suggesting that MAL is an itinerant protein cycling between the TGN and the plasma membrane (Puertollano and Alonso, 1999). Taken together, these data indicate that MAL plays a critical role in the efficient incorporation of the exocytic uroplakin-delivering vesicles into the urothelial apical membrane.

Thus our data indicate that MAL does not play a role in the apical sorting of uroplakins, gp114, and gp135 and the faithful incorporation of uroplakins into fusiform vesicles that can fuse with the apical surface of umbrella cells (Figure 3F). Our gp114 result is apparently inconsistent with an early report that this protein was mistargeted to the basolateral membrane (Cheong et al., 1999); the reason arrow in G) and led to the formation of numerous multivesicular bodies (I; also see Figure 5), asterisk, fusiform vesicle; D, desmosome; I, intermediate cell; L, lumen; MVB, multivesicular body; TJ, tight junction; U, umbrella cell. Bars, 50 µm (C) and 0.5 µm (all others).
for this discrepancy is unclear. In sum, our MDCK and in vivo data are consistent with each other and strongly suggest that the main in vivo function of MAL in mouse urothelial umbrella cells is to facilitate the incorporation of exocytic vesicles into the apical surface membrane, after they have been tethered to the apical zone by a MAL-independent mechanism. Additional studies are needed to determine whether MAL, a compact, highly hydrophobic, four-transmembrane-domain membrane protein (Magal et al., 2009), may function as one of the effectors that embed and regulate the soluble N-ethylmaleimide-sensitive factor attachment protein receptor–Sec1/Munc18-like or other related fusion machinery (Sudhof and Rothman, 2009).

Possible mechanisms for the apical targeting of uroplakins

Existing data suggest that apical sorting may be facilitated by the coalescence of nanoscale assemblies of lipid rafts into larger, stabilized platforms that can function in signal transduction and membrane trafficking, and that this coalescence process may be promoted by caveolin oligomers, luminal lectins, and other apical sorting receptors (Fullekrug and Simons, 2004; Rodriguez-Boulan et al., 2005; Vagin et al., 2009; Simons and Gerl, 2010). This process has also been shown to be facilitated by protein oligomerization (Paladino et al., 2004, 2007). Our finding that some of the uroplakins are associated with lipid rafts (Figure 7, F and G; Khandelwal et al., 2010), coupled with the strong propensity of uroplakins to polymerize forming heterodimers, heterotetramers, 16-nm particles, and two-dimensional (2D) crystals (Tu et al., 2002; Hu et al., 2005, 2008), suggests that these may provide dominant signals for the apical sorting of uroplakins, although Rab27b (Chen et al., 2003) and Rab11a (Khandelwal et al., 2008) may also contribute to or modulate this process.

It is interesting that in the absence of MAL expression uroplakins can still form normal-looking fusiform vesicles, indicating that MAL is not required for fusiform vesicle formation. However, these MAL-negative vesicles seem to be less efficient in fusing with the apical membrane (Figure 5, D and E; also see Figure 3E). This result contrasts with the case of some other apical proteins examined so far, which, when MAL was depleted, became retained in the Golgi (Cheong et al., 1999; Martin-Belmonte et al., 2001). MAL therefore appears to facilitate the delivery of the transport vesicles at the TGN level for some type of cargoes, but it functions only in the final stage of apical incorporation of the uroplakin storage vesicles.

The strong propensity for the uroplakins to polymerize forming 2D crystals even at an early stage of assembly leads to the clean separation of uroplakins and MAL into the plaque and hinge areas of the fusiform vesicle, respectively (Figure 6A). This suggests that MAL may not interact directly with uroplakins and that it is unlikely that uroplakin–MAL interaction per se plays a role in the apical sorting of uroplakins (cf. Tall et al., 2003).

The formation of membrane subdomains has been implicated in signal transduction and membrane trafficking. However, these subdomains are usually quite small and heterogeneous, exhibiting a spectrum of lipid and protein composition and properties, thus complicating their study (Jacobson et al., 2007; Coskun and Simons, 2010; Lingwood and Simons, 2010). For example, tetraspanins (to which uroplakins la and lb belong) form membrane microdomains that are distinguishable from typical “rafts” in their detergent resistance and protein composition (Hemler, 2003; Wright et al., 2004; Le Naour et al., 2006; Lazo, 2007; Yanez-Mo et al., 2009). Uroplakin proteins are exceptional in that they form huge, well-characterized membrane subdomains that can reach 500–1000 nm in diameter, in this case forming 2D crystalline plaques with well-defined protein and probably lipid composition (Lingwood and Simons, 2010; Simons and Gerl, 2010).

Roles of MAL in regulating the apical delivery of uroplakins in the polarized urothelial umbrella cells: a model

Taken together, our data suggest a model in which MAL is an integral part of a class of exocytic vesicle that transports the uroplakin cargoes to the apical surface of mouse urothelium (Figure 8). This model has the following salient features:

1) Normal mouse urothelial umbrella cells (Figure 8A). In this case, the newly synthesized uroplakins are probably enriched in the MAL (green arrowheads)–containing lipid raft subdomains of the TGN. These uroplakin/MAL subdomains are pinched off, forming uroplakin-delivering, immature discoidal vesicles containing scattered or loosely aggregated uroplakin particles (Severs and Hicks, 1979; Hudoklin et al., 2011). Homotypic fusion and retrograde transport of the nonuroplakin domains lead to the expansion of the 2D crystals of uroplakins and progressive decrease of the MAL-to-uroplakin ratio, culminating in the formation of a mature fusiform vesicle consisting of two large 2D crystals of uroplakins interconnected by MAL-positive, uroplakin particle–free hinge

FIGURE 5: MAL knockout resulted in an accumulation of fusiform vesicles, whereas MAL overexpression led to the partial replacement of fusiform vesicles by multivesicular bodies. Transmission electron microscopy of the wild-type (A, B), MAL-knockout (C–E), and MAL-overexpressed (F–H) mouse bladder urothelium. Note in C–E the accumulation of the uroplakin-delivering fusiform vesicles and in F–H the partial replacement of the fusiform vesicles by the degradative multivesicular vesicles. Bars, 10 µm (B, D, G) and 2 µm (E, H).
these vesicles, which are enriched in a Sarkosyl-insoluble fraction, confirmed the presence of a large amount of MAL (unpublished data). These data suggest that the small vesicles in uroplakin-deficient urothelial cells (Figure 7, B–E) are similar to the early discoidal vesicles without their uroplakin cargo and that MAL is an integral protein component of the uroplakin-delivering vesicles in normal umbrella cells (Figure 7, B–E).

b) Segregation of MAL from the uroplakins. Although MAL is relatively uniformly distributed in the immature discoidal vesicles (Figure 7, D and E), it is progressively excluded from the expanding 2D crystals of uroplakins, so that in mature fusiform vesicles it is associated exclusively with the uroplakin-free hinge areas (Figure 6, B–E). MAL is also associated with the hinge areas of the apical surface membrane (Figure 6, B–E). The fact that the plaque-associated hinge areas, like the plaques, can survive detergent treatment (Liang et al., 2001) explains why MAL copurified (Figure 1B) and cofloated (Figure 7, F and G) with uroplakins.

c) Hinge area as a possible site of fusion. The fact that MAL is associated with the (uroplakin particle-free) hinge areas of not only the fusiform vesicles (Figure 6, B–E) but also those of the apical surface (Figure 6, F and G) raises the possibility that the MAL-enriched hinge areas are involved in the fusion between the fusiform vesicles and the urothelial apical surface (Figure 6H). The mechanism by which MAL facilitates the fusion of the exocytic vesicle with the apical membrane is unclear, but it may involve a MAL and related proteins for vesicle trafficking and membrane link (MARVEL) domain, which is found in MAL, physins, gyrius, and occludin families (Sanchez-Pulido et al., 2002). These MARVEL-containing proteins may function in cholesterol-rich membrane opposition events in a variety of cellular processes (Sanchez-Pulido et al., 2002). The accumulation of fusiform vesicles in urothelial cells from MAL-knockout mice is reminiscent of that of prefusion complexes in mutants of the MARVEL domain–containing Singles Bar protein, which is required for the prefusion complex of myoblasts to progress to fusion in Drosophila embryos (Estrada et al., 2007).

d) The uroplakin-delivering fusiform vesicles can be regarded as a form of storage vesicles whose fusion with the apical surface may be up-regulated by the mechanical distention of the bladder (Lewis and de Moura, 1982; Truschel et al., 2002; Khandelwal et al., 2009; Wu et al., 2009).
of the endocytosed uroplakins can be recycled (hence the question marks in Figure 8A).

2) MAL-depleted urothelial cells (Figure 8B). Because the MAL-depleted urothelial umbrella cells can still make fusiform vesicles and the apical surface of such cells is covered by uroplakin plaques (Figure 5, D and E), MAL is clearly not required for fusiform vesicle formation and for their incorporation into the apical surface. However, MAL facilitates the fusion of the exocytic vesicles with epithelial apical surface, so that even though in its absence this fusion can still occur, it proceeds with a lower rate (Figure 3E), leading to the accumulation of fusiform vesicles (Figure 5, D and E; highlighted in a dashed circle in Figure 8B) and an increase in uroplakin content (Figure 4, A and B).

3) MAL-overexpressing urothelial cells (Figure 8C). MAL overexpression facilitates the apical incorporation, and subsequent endocytic degradation, of uroplakins, thus explaining the observed decrease in fusiform vesicles, accumulation of the multivesicular bodies (Figure 5, G and H; highlighted in a dashed circle in Figure 8C), and decreased uroplakin content (Figure 4, A and B).

Concluding remarks
Our results enabled us to dissect the apical targeting of uroplakins into two phases—the apical sorting process at the TGN level and the final incorporation of the apically targeted, uroplakin-delivering vesicles into the apical surface. We showed that MAL does not play a role in the apical sorting of uroplakins at the TGN level. Instead, it plays an important role in facilitating the subsequent incorporation of the uroplakin-delivering exocytic vesicles into the urothelial apical membrane. Although our data are based on urothelium and cultured MDCK renal cells, MAL is present near or on the apical surface of a wide range of polarized epithelial cells, including those of the respiratory, gastrointestinal, and genitourinary tracts, and in exocrine and endocrine glands such as thyroid and pancreas (Marazuela et al., 2003). In addition, MAL overexpression can lead to abnormal apical membrane formation in cultured thyroid cells (Martin-Belmonte et al., 1998, 2000) and in kidney and stomach epithelia in transgenic mice (Frank et al., 2000; Carmosino et al., 2010a). These results raise the possibility that MAL may play a similar role in facilitating the incorporation of exocytic vesicle into the apical surface of a wide range of specialized epithelial cells.
Uroplakin purification

Urothelium scraped from female Swiss Webster mice (8–12 wk) was homogenized in buffer A (10 mM 4-(2-hydroxyethyl)-1-piperazin-1-iumethanesulfonic acid, pH 7.5; 1 mM EDTA; 1 mM ethylene glycol tetraacetic acid; 1 mM phenylmethylsulfonyl fluoride), loaded onto a 1.6 M sucrose cushion in the same buffer, and centrifuged at 16,000 rpm for 25 min at 4°C in a Beckman Instruments (Palo Alto, CA) SW28 rotor. The crude membranes concentrated at the interface were isolated, washed with buffer A, treated with 2% Sarkosyl in buffer A for 10 min at 25°C, and pelleted, resulting in Sarkosyl-insoluble urothelial plaques (asymmetric unit membranes [AUMs]; Wu et al., 1994; Liang et al., 2001; Zhou et al., 2001).

Deglycosylation and immunoblotting

For endoglycosidase H (Endo H) digestion, urothelial AUM proteins were denatured in 0.5% SDS and 1% β-mercaptoethanol at 25°C for 10 min, made to contain 50 mM sodium citrate (pH 5.5), and incubated with Endo H at 37°C for 1 h (complete deglycosylation) per manufacturer’s instructions (New England BioLabs, Beverly, MA). For Endo F digestion, sodium phosphate (pH 7.5) and NP-40 were added to the denatured proteins to a final concentration of 50 mM and 1%, respectively, and the mixture was incubated with Endo F. SDS–PAGE and immunoblotting were done as described (Liang et al., 2001). Briefly, samples were subjected to SDS–PAGE in 17% acrylamide gels under reducing conditions and transferred to nitrocellulose membranes. After blocking with 5% nonfat dry milk and 0.05% Tween 20 in phosphate-buffered saline (PBS), blots were incubated with the primary antibody, washed, incubated for 1 h with goat anti–mouse or anti–rat immunoglobulin G (IgG) antibodies coupled to horseradish peroxidase, washed extensively, and developed using an enhanced chemiluminescence Western blotting kit (Amersham Pharmacia Biotech, Uppsala, Sweden).

N-terminal protein sequencing

Proteins separated by SDS–PAGE were transferred onto a polyvinylidene fluoride membrane and stained with Coomassie brilliant blue for 1 min. After destaining in 50% methanol and extensive washing in water, protein bands were excised and cut into small pieces. Amino-terminal sequencing was carried out by Edman degradation (Vogel, 2009; Khandelwal et al., 2010) and the accumulation of multivesicular bodies (in dashed circle). See the text for details.

MATERIALS AND METHODS

Antibodies and tissues

Rabbit antiserum to individual uroplakins Ia, Ib, II and IIIa, and mouse monoclonal antibody AU1 against UPIIIa, have been described (Liang et al., 2001). Rat monoclonal antibody 2E5 to dog MAL was also previously described (Puertollano et al., 1999). Other antibodies included the following: mouse monoclonal antibody to keratin K5 and goat anti–MAL (Santa Cruz Biotechnology, Santa Cruz, CA); mouse anti–E-cadherin and rat anti–ZO-1 (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA); mouse monoclonal antibody to EEA1 and GM 130 (BD Bioscience, San Jose, CA); mouse monoclonal antibody to influenza hemagglutinin (kindly provided by David Sabatini, New York University School of Medicine, New York, NY); and mouse monoclonal antibody to gp135 (kindly provided by G. Ojakian, State University of New York Downstate Medical Center, Brooklyn, NY). All mouse tissues were harvested from adult mice 8–16 wk old.

Uroplakin purification

Urothelium scraped from female Swiss Webster mice (8–12 wk) was homogenized in buffer A (10 mM 4-(2-hydroxyethyl)-1-pipera-
same tube by addition of 0.5 U of Taq DNA polymerase and 10 pmol of the primers. The full-length cDNAs of mouse uroplakins and MAL cDNA were obtained from mouse bladder total RNA by RT-PCR and subcloned into the pCDNA3 vector, and the constructs were verified by sequencing. The correct plasmids were amplified in bacteria strain Top10 and purified with Maxi Prep Kit (Qiagen, Valencia, CA).

**Biotinylation of mouse urothelial surface**

Female Swiss Webster mice (8–12 wk) were anesthetized under 2% isoflurane in oxygen, catheterized using a PE10 polyethylene tubing, and their bladders washed with PBS. Seventy-five microliters of sulfo-N-hydroxysuccinimide (NHS)-LC-biotin solution in PBS (1 mg/ml) were injected into bladder lumen over a course of 5 min. After 15 min, the bladder was rinsed with 75 µl of PBS and immediately injected with 75 µl of 50 mM lysine solution in PBS, which was left in the bladder for 15 min to quench the biotinylation reaction. Finally, the bladder was rinsed with PBS and fixed in situ with 75 µl of 10% formalin/PBS and sectioned, and the biopsy was visualized using fluorescein isothiocyanate (FITC)-conjugated streptavidin (green fluorescence; nuclei counterstained in blue with 4′,6-diamidino-2-phenylindole).

**Cell culture, transfection, and immunofluorescence staining**

MDCK cells (strain II; American Type Culture Collection, Manassas, VA) were cultured in DMEM (Life Technologies, Rockville, MD) containing 15% fetal bovine serum (HyClone Laboratories, Logan, UT), penicillin (100 U/ml), and streptomycin (100 µg/ml) in a 5% CO₂ atmosphere. When grown on filters, 2 × 10⁵ cells were seeded on polycarbonate membranes (diameter, 0.4 µm pore size; Transwell chambers; Costar, Cambridge, MA). The plasmids containing cDNA or siRNA were transfected using NuPore size; Transwell filters; Costar, Cambridge, MA). The MDCK cells were seeded on coverslips or Transwell filters, or tissue sections, were fixed and used for indirect immunofluorescence staining. Primary antibodies in 3% or Transwell filters, or tissue sections, were fixed and used for Western blot with appropriate antibodies.

**Electron microscopy**

For transmission electron microscopy, mouse bladders were cut into small pieces (<1 mm²), fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4), postfixed with 1% (wt/vol) osmium tetroxide, and embedded in EMBed 812 (Electron Microscopy Sciences, Hatfield, PA) as described (Liang et al., 2001). For immuno–electron microscopy, mouse bladders were fixed for 4 h at 4°C in a freshly prepared solution containing 3% paraformaldehyde, 0.1% glutaraldehyde, and 4% sucrose. A 0.1 M sodium cacodylate buffer (pH 7.4) was used for LK4M embedment, and 0.1 M PBS (pH 7.4) was used as buffer for cryoimmunolabeling. Goat anti–mouse IgG conjugated with 10- or 15-nm gold particles (Amersham Life Science, Arlington Heights, IL), Nanogold conjugated anti–goat Fab, and HQ silver enhancement kit (Nanoprobes, Yaphank, NY) were used for antigen detection (Liang et al., 2001; Romih et al., 2005). Stained grids were examined using a Philips CM-12 electron microscope (FEI, Eindhoven, Netherlands) and photographed with a Gatan (4k x 2.7k) digital camera (Gatan, Pleasanton, CA).

**siRNA**

MAL siRNA and control sequences were designed based on the algorithm variable at https://maidenigner.invitrogen.com/rnaiexpress/rnaIExpress.jsp?cid=fl-RNAIEXPRESS. Three candidate double-stranded stealth RNA targeting dog MAL were designed and synthesized by Invitrogen: si200 (5′-CAG CCC UGC UUG UCC UGU ACA UAA U-3′), si269 (5′-CCU ACC ACU GUA UUG CUG CCC UGU U-3′), and si411 (5′-GCU GAU CGA UGU CAU CCA UGA GUU U-3′), plus a control scrambled sequence (5′-CCU UCA CUA UGCU CGC GCC AGU U-3′). The efficiency of MAL knockdown was measured by Western blot with monoclonal antibody anti-MAL 2E5 (Supplemental Figure S3).


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