Clathrin-mediated Endocytosis of the Epithelial Sodium Channel

ROLE OF EPSIN

Here we present evidence that the epithelial sodium channel (ENaC), a heterotrimeric membrane protein whose surface expression is regulated by ubiquitination, is present in clathrin-coated vesicles in epithelial cells that natively express ENaC. The channel subunits are ubiquitinated and co-immunoprecipitate with both epsin and clathrin adaptor proteins, and epsin, as expected, co-immunoprecipitates with clathrin adaptor proteins. The functional significance of these interactions was evaluated in a Xenopus oocyte expression system where co-expression of epsin and ENaC resulted in a down-regulation of ENaC activity; conversely, co-expression of epsin sub-domains acted as dominant-negative effectors and stimulated ENaC activity. These results identify epsin as an accessory protein linking ENaC to the clathrin-based endocytic machinery thereby regulating the activity of this ion channel at the cell surface.

The epithelial Na⁺ channel (ENaC)² is expressed in absorptive tissues that establish low luminal Na⁺ concentrations. Abnormalities of this channel contribute to the pathophysiology of a number of diseases including Liddle’s syndrome, pseudo-hypaldosteronism and cystic fibrosis (1–3). ENaC is a heterotrimeric channel, composed of three homologous subunits, α, β, and γ (4). The channel complex is inefficiently processed with less than 20% of newly synthesized subunits reaching the apical membrane of endogenously expressing epithelia (3, 5). The apical membrane stability of these channels is a subject of dispute but it is widely agreed that ENaC is retrieved from the apical membrane by a process involving ubiquitination (6). Early studies in oocytes demonstrated that mutation of a C-terminal PY motif, or co-expression of a dominant negative dynamin, resulted in increased ENaC activity, consistent with regulation of surface expression by clathrin-mediated endocytosis (7). Subsequently it was recognized that the ubiquitin-protein ligase Nedd4-2 binds to the consensus PPXY (PY) motifs in the cytoplasmic tails of ENaC subunits via its N-terminal ubiquitin interaction motifs; CCM, clathrin-coated vesicles; UIM, ubiquitin interaction motifs; CCM, clathrin-coated vesicles; MES, 4-morpholineethanesulfonic acid; IP, immunoprecipitate; GST, glutathione S-transferase; MBS, modified Barth’s saline; BSA, bovine serum albumin.

Clathrin-mediated endocytosis is a process by which specific membrane proteins are internalized from the plasma membrane by eukaryotic cells. Target proteins bind through classic motifs to adaptor proteins that link the cargo to clathrin lattices that deform the cell membrane to form a bud and ultimately an endocytic vesicle (8). Membrane proteins that are modified by attachment of one or more ubiquitin molecules appear to be internalized by signals unlike the classic linear internalization motifs and may require binding to accessory proteins that link them to clathrin adaptors (9). Proteins of the epsin family appear to be excellent candidates to function as adaptor coupling proteins because they contain ubiquitin interaction motifs (UII) as well as clathrin binding motifs (10, 11), and epsin has been shown to down-regulate ENaC activity when co-expressed in Chinese hamster ovary cells (12). Epsin orthologs in Drosophila and Caenorhabditis elegans target monoubiquitinated ligands of the Notch pathway for endocytosis (13–15). However, direct experimental evidence for a role of epsins linking ubiquitinated cargo to clathrin-coated vesicles in mammalian cells is lacking (9). The current studies were undertaken to examine the possibility that ENaC internalization involves clathrin-mediated endocytosis and to determine whether epsin is involved in linking ubiquitinated ENaC to clathrin adaptors.

MATERIALS AND METHODS

Reagents—Rabbit polyclonal antibodies to α and β ENaC were kindly provided by Dr. Kathi Peters (University of Pittsburgh; α ENaC) and Dr. Mark Knepper (Laboratory of Kidney and Electrolyte Metabolism, NHLBI, National Institutes of Health, Bethesda MD; β ENaC) and their specificity in CCD cells has been described (16). Chicken polyclonal antibody to γ ENaC has been described (17). γ ENaC bands visualized by this antibody in CCD cells were competed by incubation with immunizing peptide (not shown). Mouse monoclonal antibody to ubiquitinated proteins (clone FK2) was purchased from Biomol. Mouse monoclonal antibody to α adaptin was purchased from BD Biosciences, and EE1 rabbit polyclonal antibody was a gift from Dr. Silvia Corvera. Antibodies to heavy chain clathrin, β and γ adaptin and epsin have been previously described (18). Rabbit polyclonal antibody to the N terminus of μ2 was the generous gift of Dr. Juan Bonifacino (NIH, Bethesda, MD). βR564X ENaC mutant was generated as previously described (19). The full-length epsin 1 cDNA in pBluescript was a generous gift from Pietro De Camilli (Yale University School of Medicine, New Haven, CT). The epsin DPW domain (residues Arg²⁵⁹–Asp⁴⁰⁷ with a subsequent TAA stop codon) was amplified from this by PCR and then inserted into pcDNA3.1myc using the EcoRI and XbaI sites. The ENTH domain construct (Met¹–Ala¹⁶³) was prepared by introduction of an Ala¹⁶⁴stop mutation in full-length epsin 1 inserted into pcDNA3.1myc as for the DPW domain. The proteins encoded by the pcDNA3.1myc vector have...
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an N-terminal myc epitope tag. GST-epsin 1-(1–407) has been previously described (20). For construction of ENaC-ubiquitin conjugates, a plasmid containing three tandem ubiquitin (gift from G. Lukacs, Toronto) was amplified using unique primers containing a BspHI site on the 5 primer. The product was cloned into pTopo-Blunt II (Invitrogen). The ubiquitin containing fragment was excised using BspHI and Xhol (in linker region) and cloned in-frame to the carboxyl end of α-ENaC that had been cleaved using Ncol and Xhol. Cloning into the Ncol site on α-ENaC results in the deletion of the final two amino acids of the native protein. For both β and γ ENaC a KpnI site was introduced before the termination codon and the ubiquitin construct was ligated into the KpnI site and a unique downstream restriction enzyme site.

Lysine to arginine mutants of α and γ ENaC (αK6–13R and γK6–13R) (K6–13R is representative for K6R,K8R,K10R,K12R,K13R) were generated by substituting specific lysine residues with arginines using PCR-based mutagenesis as described (6). All constructs were verified by automated dyeoxygenucleotide sequencing.

The mpkCCD14 cells (kindly provided by Alain Vandewalle and Marcelle Bens) were grown in flasks, tissue culture dishes, and Tranwell porous supports. Growth medium was composed of equal volumes Dulbecco’s modified Eagle’s medium and Ham’s F-12, 60 mM sodium selenate, 5 μg/ml transferrin, 50 mM dexamethasone, 1 mM triiodothyronine, 10 ng/ml epidermal growth factor, 5 μg/ml insulin, 20 mM d-glucose, 2% (v/v) fetal calf serum, and 20 mM HEPES, pH 7.4, at 37 °C in 5% CO2, 95% air atmosphere. For all experiments the CCD cells were resuspended in 0.2–0.5 ml of RIPA buffer, frozen in liquid nitrogen and pelleted by centrifugation at 167,000 g in a TST 60.1 rotor at 4 °C and the supernatant was saved. The supernatant was resuspended in 0.2–0.5 ml of isolation buffer and sheared through a 22-gauge needle 20 times. Following a low speed spin, the post-nuclear supernatant was pipetted 10 times, then lysed by passing through a 22-gauge needle 20 times. A clear centrifuge tube (Seton Scientific, Sunnyvale, CA). 1.5 ml of 35% sucrose, 10 mM MES-NaOH, pH 6.5, 100 mM KCl, 1 mM EGTA, 0.5 mM MgCl2, 0.02% NaN3, with protease inhibitors, using a Dounce homogenizer, then sonicated by 3 bursts of 10 s on ice. The homogenate was centrifuged at 17,800 × g for 20 min using Sorvall T865 rotor and Sorvall Discovery 90 centrifuge at 4 °C and the supernatant was saved. The supernatant was sedimented at 56,000 × g for 1 h. The pellet was resuspended in 1.7 ml of isolation buffer and sheared through a 22-gauge needle 20 times. The redissolved pellet was added on top of an equal volume of 8% sucrose in isolation buffer made in D2O and centrifuged at 115,800 × g for 2 h (S120AT2–0112 rotor; Sorvall RC M120EX centrifuge). The final pellet represented the CCV preparation.

Clathrin-coated Vesicle (CCV) Isolation—CCVs were isolated by a modification of the method of Metzler et al. (21). CCD cells cultured on 42.2-cm2 filter supports were washed 3 times with ice-cold phosphate-buffered saline on ice. Cells were scraped and homogenized in isolation buffer containing 10 mM MES-NaOH, pH 6.5, 100 mM KCl, 1 mM EGTA, 0.5 mM MgCl2, 0.02% NaN3 with protease inhibitors, using a Dounce homogenizer, then sonicated by 3 bursts of 10 s on ice. The homogenate was centrifuged at 17,800 × g for 20 min using Sorvall T865 rotor and Sorvall Discovery 90 centrifuge at 4 °C and the supernatant was saved. The supernatant was sedimented at 56,000 × g for 1 h. The pellet was resuspended in 1.7 ml of isolation buffer and sheared through a 22-gauge needle 20 times. The redissolved pellet was added on top of an equal volume of 8% sucrose in isolation buffer made in D2O and centrifuged at 115,800 × g for 2 h (S120AT2–0112 rotor; Sorvall RC M120EX centrifuge). The final pellet represented the CCV preparation.

Channel Expression in Xenopus Oocytes—Sequences for all wild type and mutant constructs were confirmed by automated DNA sequence analysis performed at the University of Pittsburgh DNA sequencing facility. Complimentary RNAs (cRNA) for wild type and mutant constructs were prepared using a cRNA synthesis kit employing T3 RNA polymerase (mMESSAGE mMACHINE, Ambion Inc., Austin, TX). Xenopus oocytes (stage V–VI) were pretreated with 2 mg/ml collagenase (type IV) in calcium-free saline solution. Murine ENaC cRNAs (1–3 ng/subunit in 50 nl of H2O) were microinjected into all oocytes. Oocytes in the experimental group were additionally injected with 5 ng of cRNA of epsin, the ENTH domain of epsin, or the DPW domain. All oocytes were incubated at 18 °C in modified Barth’s saline (88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO3, 0.3 mM Ca(NO3)2, 0.41 mM CaCl2, 0.82 mM MgSO4, 15 mM HEPES-NaOH, pH 7.2) supplemented with 10 μg/ml sodium penicillin, 10 μg/ml streptomycin sulfate, and 100 μg/ml gentamycin sulfate. Whole cell currents were measured 24–46 h after cRNA injections. To determine whether the effects of epsin on ENaC were specific, we examined ROMK expressing oocytes (22) with and without epsin. Wild type ROMK cRNA (2 ng) was injected with or without epsin cRNA (5 ng).

Whole Cell Current Measurements—A two-electrode voltage clamp technique was used as previously described (22). Whole cell inward amiloride-sensitive currents were measured in control oocytes expressing αβγ ENaC alone or experimental oocytes expressing αβγ ENaC + epsin, ENTH domain, or DPW domain, using a DigiData 1200 interface (Axon Instruments, Foster City, CA) and a TEV 200A Voltage Clamp amplifier (Dagan Corp., Minneapolis, MN). Data acquisition and analysis were performed using pClamp 7.0. Amiloride-sensitive currents were defined as the difference of the current in the absence and presence of 0.1 mM amiloride. Membrane potentials were clamped from −140 to +60 mV in 20-mV increments with duration of 900 ms. Currents were measured at a holding potential of −100 mV, 600 ms after initiation of...
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To determine whether ENaC subunits were present in CCV, we employed a standard method (21) for isolating these vesicles from a continuous line of mouse cortical collecting duct cells that express the channel endogenously (23, 24). CCVs prepared from mpkCCD14 (CCD cells) were first characterized in terms of enrichment of coat proteins and adaptors. A typical example of such a preparation is shown in Fig. 1A. Compared with the post-nuclear supernatant and crude microsomes, CCV were enriched in clathrin heavy chain, and adaptors found at Golgi (γ adaptin) and the plasma membrane (μ2). Typically, epsin was present but not markedly enriched in CCVs. Fig. 1B demonstrates that this CCV preparation was highly enriched in all three ENaC subunits. The pattern of subunit enrichment is interesting and deserves comment. We have recently observed that ENaC subunits expressed alone produce single bands on SDS gels by immunoblot: α at 95 kDa, β at 96 kDa, and γ at 93 kDa. However, when expressed together, a second band appears for each subunit (65 kDa for α, 110 kDa for β, and 75 kDa for γ). These appear to represent the processed forms of the subunits as they exhibit complex N-glycans and the α and γ subunits appear to undergo proteolytic processing (25). Both mature and immature forms of ENaC appear at the apical membrane (26). The experiments describing ENaC subunit processing were carried out in Madin-Darby canine kidney cells overexpressing double epitope-tagged subunits (25). In endogenously expressing epithelia, shorter forms of γ but not α ENaC have been reported following stimulation of ENaC activity by aldosterone (27). Complex glycosylation of ENaC has been reported in A6 cells, although with full-length γ ENaC (28). In CCD cells, CCVs appear to be enriched in the mature forms of β and γ ENaC, but only full-length α is demonstrated.

To demonstrate endocytosis of the channel, we have taken advantage of the relatively rapid insertion and retrieval of ENaC in response to elevated cAMP. In CCD epithelia, forskolin stimulates rapid and parallel increases in ENaC currents and membrane capacitance (t1/2 ~ 4.5 min). Its withdrawal results in equally rapid reversal of these capacitance and current responses, which are blocked by chloroquine, consistent with endocytic retrieval of ENaC (16). We have examined the appearance of α ENaC in early endosomes following forskolin withdrawal. Early endosomes were identified by their internalization of horseradish peroxidase (Fig. 2A) and they were isolated on a discontinuous sucrose gradient as previously described (17). Fractions enriched in EEA1, a marker for early endosomes, were enriched in the three ENaC subunits as well (Fig. 2B). Following forskolin withdrawal, there is a rapid increase in the α subunit of ENaC in early endosomes over a 10-min time course, which is typical of ENaC current decay kinetics (16) (Fig. 2C). These data indicate that ENaC traffics through an early endosomal compartment following its endocytosis from the apical membrane within CCVs.

To explore the relationship of ENaC subunits to adaptor proteins, co-immunoprecipitation studies were performed. CCD cell lysates were preincubated with horseradish peroxidase (HRP) on the apical membrane and allowed to take up the enzyme at 37 °C for 10 min. Cells were then gently lysed as described and the post-nuclear supernatant centrifuged on the sucrose gradient. Sequential fractions were taken from the top of the gradient and assayed for horseradish peroxidase. A sharp increase in horseradish peroxidase was noted in fraction 4, which corresponded to the interface between the 25 and 35% sucrose (n = 3). Similarly, appearance of α ENaC in EEA1 endosomes following forskolin withdrawal. CCD cells were incubated with 10 μM forskolin, which induced a rapid increase in I₄, and apical membrane capacitance consistent with exocytotic insertion of ENaC (16). Washout of forskolin results in a rapid and parallel decline in I₄, and capacitance consistent with retrieval of ENaC via endocytosis with a t₁/2 of 4.5 min. EEA1 fractions from discontinuous gradients were isolated from CCD cells at the time of forskolin removal (0) and at 2, 5, and 10 min following removal. Equivalent amounts of protein were loaded from each time point, separated by SDS-PAGE, and probed for EEA1 and α ENaC. As shown in the upper panel, amounts of EEA1, a control for loading, stayed relatively constant or decreased slightly. Amounts of α ENaC detected in early endosomes progressively increased over the time course of current decline following forskolin withdrawal (n = 3).
subjected to immunoprecipitation with antibodies specific for α, β, and γ ENaC and analyzed for the presence of epsin by immunoblot. Fig. 3A shows that epsin was efficiently co-immunoprecipitated by all three ENaC subunits. Furthermore, the subunits of ENaC were found to be ubiquitinated under these assay conditions. Fig. 3B demonstrates that both α and β ENaC when immunoprecipitated from CCD lysates can be detected with antibodies specific for ubiquitin. In addition, immunoprecipitations performed with anti-ubiquitin are positive for both α and β ENaC when probed with ENaC-specific antibodies. IP with anti-ubiquitin also immunoblots with epsin antibody. C, CCD cell lysate was immunoprecipitated with epsin antibody and immunoblotted with antibody to α-adaptin. D, CCV preparation was solubilized and IP with μ2 antibody and probed for α and β ENaC. E, negative controls. We incubated beads alone, pre-immune serum (PS), or α ENaC antibody with cell lysate then blotted for α ENaC. No bands are apparent with beads alone or nonspecific IgG (n = 3). WB, Western blot.

in vitro translated α, β, and γ ENaC, or each ENaC subunit fused to three tandem ubiquitins in the presence of [35S]methionine and incubated with GST alone, GST-UIM, or GST-epsin, then pulled down the GST constructs with glutathione-Sepharose beads. Interactions between translated subunits and fusion proteins will shift the translated proteins from supernatant to pellet. As shown in Fig. 4, each ENaC subunit is found in pellet when conjugated to ubiquitin. There is a small amount of ENaC alone that is bound to GST-epsin, but the major shift of subunits from supernatant to pellet is seen with ubiquitinated forms. These experiments demonstrate that ENaC interacts with epsin strongly when ubiquitinated and that the UIM domain of epsin is sufficient for this interaction.

To examine the significance of the interaction between ENaC and epsin, the Xenopus oocyte expression system was employed. Although previous studies have demonstrated that ENaC activity in oocytes is negatively regulated by Nedd4-2, we examined this first as a baseline for further experiments. All three ENaC subunits were expressed, with or without Nedd4-2, and ENaC activity was measured as amiloride-sensitive current using the two-electrode voltage clamp as described (22). Fig. 5A demonstrates that co-expression of Nedd4-2 markedly down-regulated ENaC activity in oocytes as previously shown (1, 6). We next examined the effect of epsin on ENaC activity in oocytes by expressing αβγ ENaC with or without full-length epsin. As shown in Fig. 5B, epsin down-regulated ENaC activity almost to the extent seen with expression of Nedd4-2, consistent with a role for epsin in promoting endocytosis of the channel. The effect of epsin on ENaC surface expression was measured using a FLAG-tagged β ENaC coexpressed with wild-type αγ ENaC. We have previously shown that this construct behaves similarly to wild type αβγ ENaC in oocytes (22). As shown in Fig. 5C, epsin co-expression decreased ENaC surface expression to a similar degree as it down regulated the amiloride-sensitive current (Fig. 5B). Thus, the effect of epsin on ENaC activity is totally explained by decreased ENaC expression at the plasma membrane. To determine whether ENaC ubiquitination was required for the action of epsin on ENaC activity in oocytes, we coexpressed epsin with a truncation mutant of β ENaC along with wild type α and γ ENaC. Such mutations of C-terminal β
Epsin eliminates the binding motif for the third WW domain of Nedd4-2 to βENaC and blocks ubiquitin-dependent down-regulation of the channel (6, 31). As shown in Fig. 5D, epsin had no effect on the activity of αβγENaC constructs when co-expressed in Xenopus oocytes. This result is consistent with the notion that epsin-mediated down-regulation of ENaC activity is dependent on interaction of the channel with Nedd4-2. To further demonstrate the requirement for ubiquitination in the ENaC-epsin interaction, we coexpressed wild type ENaC with a mutant epsin in which the UIM motif had been deleted. As shown in Fig. 5E, deletion of the epsin UIM motif abolished the epsin-mediated reduction of ENaC activity. We also examined the lysine to arginine mutations in N-terminal α and γ ENaC that Staub and colleagues (6) described as inhibiting ubiquitination of the channel and increasing surface expression and activity of ENaC in oocytes. As shown in Fig. 6A, lysine to arginine mutations in the N termini of either α or γ ENaC lead to modest increases in ENaC activity in oocytes compared with wild type channels, whereas expression of both mutant subunits along with wild type β ENaC results in more substantial enhancement of ENaC activity as described by Staub et al. (6) and pointing to the possible significance of mult ubiquitination of the channel (32, 33). If the epsin-ENaC interaction is dependent on subunit ubiquitination, then we would expect that epsin would have less effect on channels expressing lysine to arginine mutations that inhibit ubiquitination. This result is seen in Fig. 6B. As shown previously, epsin decreased the activity of wild type αβγ ENaC but had no effect on the activity of αβγENaC with K47R,K50R and K6–13R channels. As a further control we examined the effects of epsin on activity of a distinct ion channel, ROMK. ROMK activity in oocytes was unaffected by co expression of epsin (Fig. 7A).

Finally, we examined several of the domains of epsin to determine whether they would affect ENaC activity in oocytes. The central clathrin-binding domain of epsin is characterized by a clathrin box motif and multiple Asp-Pro-Trp (DPW) repeats (10) (Fig. 7B). If epsin were involved in endocytosis of the channel, this domain should act as a dominant negative and stimulate ENaC activity. The ENTH domain of epsin is a phosphoinositide binding domain thought to be important for either membrane localization of epsin, and/or to facilitate membrane curvature by its interaction with phosphoinositides (34, 35). Both domains were co-expressed with ENaC subunits and activity was compared with ENaC alone (Fig. 7C). As expected, the DPW domain acted as a dominant negative and stimulated ENaC activity, and a similar result was obtained with co-expression of the ENTH domain.

DISCUSSION

The apical membrane residence of ENaC has been shown to be primarily regulated by the ubiquitin ligase Nedd4-2 (3). This process can be
regulated by kinases that alter the interaction between Nedd4-2 and ENaC (2, 3) or by mutations of the β and γ subunits of the channel that block ubiquitination and lead to enhanced surface expression, and, in humans, hypertension (2, 31). The mechanism(s) by which ubiquitinated ENaC is internalized from the apical membrane are, however, not entirely known. Evidence from oocyte studies demonstrate that the process is dynamin-dependent, consistent with a role for clathrin-mediated endocytosis (7). The partners that link ubiquitinated cargo, such as ENaC, to clathrin-coated vesicles are a subject of considerable study and interest (8, 9). Multimodular proteins such as epsin, which have domains that interact with ubiquitin and domains that interact with clathrin adaptors, have been proposed to play such a role (10, 11), and epsin does down-regulate ENaC activity when co-expressed with ENaC in Chinese hamster ovary cells (12). We sought to determine whether ENaC surface expression might be regulated by clathrin-mediated endocytosis, and whether epsin is involved in this process.

Our results demonstrate that ENaC is present in clathrin-coated vesicles in CCD cells, and is efficiently endocytosed, consistent with previous observations that channel activity is regulated in a dynamin-dependent manner (7). ENaC is ubiquitinated, as would be expected for a protein whose surface expression is negatively regulated by ubiquitin ligase (1). There are some differences in the degree of ubiquitination of ENaC shown here for the CCD cells and that demonstrated by others in either human embryonic kidney cells overexpressing ENaC (6) or endogenously expressing A6 cells (36). These differences may be due to the different cell systems or antibodies employed or to rates of deubiquitination. Nevertheless, all studies have shown ubiquitination of multiple subunits of this channel.

FIGURE 7. Effect of epsin on ROMK activity and effect of epsin domains on ENaC activity. A, RNA for ROMK was injected into oocytes with and without full-length epsin and activity was measured at 24 h. Co-expression of epsin had no effect on ROMK activity. B, schematic of full-length epsin 1 and the ENTH and DPW domains used for oocyte injections. C, amiloride-sensitive currents from αβγ ENaC alone were compared with currents in the presence of either DPW or ENTH domains of epsin. Co-expression of each domain with ENaC resulted in a significant enhancement of ENaC activity (p < 0.005, n = 32). Statistical comparisons were made by unpaired t test, or analysis of variance followed by t test for multiple comparisons.
ENaC is a tetrameric channel composed of two α, one β, and one γ subunit. Studies by Staub and colleagues (6) demonstrated ubiquitination of α and γ ENaC expressed in human embryonic kidney cells and inhibition of ubiquitination of only γ ENaC by mutations of lysine residues in the N terminus resulted in increased surface expression due to decreased retrieval of the channel. Additional mutations to the α subunit augmented this effect, suggesting that multiubiquitination of the subunits was important to ubiquitin-dependent retrieval (see Fig. 6A). These observations leave open the question of how ubiquitination of multiple ENaC subunits leads to endocytosis that would be dominant-dependent. We show that the channel subunits interact with epsin in CCD cells, and ENaC and epsin interact with clathrin adaptors in these cells. Epsin negatively regulates channel activity in oocytes, as it does in Chinese hamster ovary cells (12) and this effect is dependent on the UIM motif of epsin. Moreover, the effect of epsin on ENaC activity can be abolished by mutations in the channel that block its ability to interact with the ubiquitin ligase Nedd4-2 or that remove the sites for its ubiquitination. Because the effect of epsin on ENaC activity was dependent both on ubiquitination of ENaC and the presence of the UIM domain of epsin, we examined the interaction in vitro between ENaC and epsin. Only ubiquitinated ENaC is seen to interact significantly with epsin, and the same result is seen with expression of the UIM domain of epsin alone. Our results are therefore consistent with previous observations that surface expression of ENaC is regulated in both a dynamin and ubiquitin-dependent manner (6, 7), but they implicate epsin as the link between ubiquitinated ENaC on the cell surface and clathrin adaptors. Our results do not rule out the possibility that some portion of ENaC internalization may also be mediated by caveolae as has been described for another ubiquitinated membrane protein, the epidermal growth factor receptor (37).

Epsin appears to have no effect of a distinct ion channel, ROMK, when co-expressed in oocytes, suggesting that the effect of epsin is not to globally stimulate endocytosis. The effects of the non-UIM domains of epsin on ENaC are also of interest. The dominant negative effect of the DPW domain of epsin is consistent with AP-2 dependence of this process, simply titrating adaptor proteins; and would be expected for any protein whose surface expression is regulated by clathrin-mediated endocytosis. The similar effect of the ENTH domain suggests that phosphoinositide interactions are important in regulating ENaC internalization. Taken together, the results are consistent with a model where epsin links multiubiquitinated cargo to adaptor complexes and regulates endocytosis.

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