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Regulation of the epithelial sodium channel (ENaC), which regulates fluid homeostasis and blood pressure, is complex and remains incompletely understood. The TIP peptide, a mimic of the lectin-like domain of TNF, activates ENaC by binding to glycosylated residues in the extracellular loop of ENaC-α, as well as to a hitherto uncharacterized internal site. Molecular docking studies suggested three residues, Val567, Glu568, and Glu571, located at the interface between the second transmembrane and C-terminal domains of ENaC-α, as a critical site for binding of the TIP peptide. We generated Ala replacement mutants in this region of ENaC-α and examined its interaction with TIP peptide (3M, V567A/E568A/E571A; 2M, V567A/E568A; and 1M, E571A). 3M and 2M ENaC-α, but not 1M ENaC-α, displayed significantly reduced binding capacity to TIP peptide and to TNF. When overexpressed in H441 cells, 3M ENaC-α subunits but not 3M or 2M ENaC-α channels. On the other hand, TIP peptide-mediated reduction in ENaC ubiquitination was similar in cells overexpressing either WT or 3M ENaC-α subunits. In summary, this study has identified a novel site in ENaC-α that is crucial for activation of the open probability of the channel, but not membrane expression, by the lectin-like domain of TNF.

The epithelial sodium channel (ENaC)6 constitutes the rate-limiting entry step in Na+ reabsorption across epithelia in colon, kidney, and lungs (1). ENaC in its native form consists of three subunits: α, β, and γ, the former of which is crucial to obtain functional channels (2). A unique fourth subunit, ENaC-δ, can also form channels with the β and γ subunits but exhibits biophysical and pharmacological properties that are divergent from those of α ENaC channels (3). ENaC subunits consist of relatively short N and C termini, linked to two membrane spanning transmembrane helices (TM1 and TM2), in which the pore and gating domains of the channel are situated, and a large extracellular loop region containing glycosylated Asn residues (4–6). ENaC function depends on the number of channels in the membrane (N), which changes according to membrane insertion or retrieval (7), as well as on the activity of individual channels, defined by their open probability, Po (8). A variety of external factors, including acidic pH, Na+, and proteases modify ENaC activity through interaction with domains

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4 The abbreviations used are: ENaC, epithelial sodium channel; TM, transmembrane; ASIC1, acid-sensing ion channel 1; PIP2, phosphatidylinositol bisphosphate; MARCKS, myristoylated alanine-rich C kinase substrate; ARDS, acute respiratory distress syndrome; TUBE2, tandem ubiquitin binding entities 2; P, open probability; Po, open probability.
in the extracellular loop regions of the channel (4, 9). Allosteric changes that can occur upon binding of extracellular Na\(^+\) were indicated using the resolved crystal structure of acid-sensing ion channel 1 (ASIC1), a related ENaC/degenerin family member, which has \(\sim\)30% homology to the ENaC-\(\alpha\) subunit (6, 10). Moreover, a recent report demonstrated that residues forming interfaces between the extracellular domains of the three ENaC subunits could affect gating of the channel (11).

ENaC expression is down-regulated by neural precursor cell expressed developmentally down-regulated protein 4-2 (Nedd4-2) upon direct binding of the WW domains to PY motifs of ENaC (12), which causes ubiquitination and removal of the channel from the membrane. Open probability of ENaC can be regulated by various mechanisms, including Na\(^+\) self-inhibition (13), proteolysis (14), laminar shear stress (15), palmitoylation of the \(\beta\) and \(\gamma\) subunits (16), and eicosanoids (17). Moreover, phosphatidylinositides in the inner leaflet of the plasma membrane are able to increase \(P_o\) and the most prominent member of these is phosphatidylinositol bisphosphate (PIP\(2\)) (18). Myristoylated alanine-rich C kinase substrate (MARCKS) protein at the plasma membrane was shown to regulate the delivery of PIP\(2\) to ENaC (19). The cytoplasmic N and C termini of the ENaC subunits represent sites of chemical modification, protein binding, and interactions with components of the plasma membrane, in response to signals that regulate ENaC function by either changing \(N_o\) or \(P_o\) of the channel. In addition, a domain in the C terminus (Glu\(^{604}\)–Cys\(^{618}\)) of ENaC-\(\alpha\), between TM2 and the PPXY motif, was shown to bind actin, indicating an important link between ENaC activity and the cytoskeleton (20–23).

In view of its vital role in the regulation of total body fluid homeostasis and in the control of blood pressure, it is important to identify mediators and mechanisms affecting ENaC expression and open probability. Because vectorial Na\(^+\) flux mediates liquid transport in the lungs, ENaC activity has to be tightly regulated. This becomes especially challenging during pulmonary infection and inflammation, as can be found during ARDS and pneumonia. Because ENaC regulates the airway surface liquid, its exaggerated activity might lead to airway dehydration, mucus stasis, and bacterial overgrowth, as can be seen in cystic fibrosis and chronic bronchitis (24–26). By contrast, ENaC hypoactivity can dramatically impair alveolar liquid clearance, which is particularly important in conditions of pulmonary edema and correlates with mortality and morbidity in patients with acute lung injury and ARDS (27). The inflammatory milieu during ARDS and pneumonia can contain significantly increased concentrations of bacterial toxins, especially upon antibiotic treatment, including LPS from G\(^-\) bacteria and pneumolysin from G\(^+\) pneumococci, both of which have been shown to negatively affect ENaC function in a PLC and PKC-dependent manner (28–31). Moreover, increased concentrations of pro-inflammatory cytokines in the alveolar space can also affect the channel. As such, the pathogenic factor TGF-\(\beta\), which can be found in significantly increased levels in ARDS patients, was shown to reduce expression of the \(\beta\) subunit of ENaC (32).

Intriguingly, the pro-inflammatory cytokine TNF was demonstrated to either reduce or increase ENaC function (33–35). We and others propose that the negative effects of TNF in acute lung injury in general and in ENaC function in particular mainly involve binding to TNF receptor 1, which in turn reduces ENaC-\(\alpha\), \(\beta\), and \(\gamma\) transcription, as well as ENaC-\(\alpha\) protein expression (36, 37). The lectin-like domain of TNF, which is spatially distinct from the receptor binding sites and which is mimicked by the 17 residue TIP peptide (also called AP301 and solnatide), activates ENaC, upon direct binding to the ENaC-\(\alpha\) subunit (29, 38). Intriguingly, our recent experimental and molecular modeling data indicated that two distinct binding sites exist for the TIP peptide in ENaC-\(\alpha\). The first one, obtained through mutational analysis in a heterologous cell system, consists of glycosylated Asn residues (Asn\(^{293}\), Asn\(^{312}\), Asn\(^{397}\), and Asn\(^{411}\)) in the extracellular loop of the subunit (38), whereas the second one is a yet unidentified site within the C-terminal domain suggested through molecular modeling using ASIC1 as a template (29). The first aim of this study was to map this second ENaC-\(\alpha\) binding site for the lectin-like domain of TNF. As the second aim, we investigated whether this site can affect the open probability or expression of the channel.

**Results**

**Mapping Residues of ENaC-\(\alpha\) Binding to the TIP Peptide—**Our recently published data have demonstrated that the TNF-derived TIP peptide, which mimics the lectin-like domain of the cytokine, requires intact cellular N-glycosylation to activate ENaC in HEK 293 and H441 cells, with N-glycan \(\alpha\)Asn\(^{511}\) being crucial (29, 38). This suggests that glycosylated residues in the extracellular loop of ENaC-\(\alpha\) are crucial for activation of the channel by TNF. However, a mutant TIP peptide in which three residues (one Thr and two Glu) crucial for ENaC activation were changed to Ala still efficiently associates with oligosaccharides to which the lectin-like domain of TNF binds, such as \(N,N'\)-diacetylchitobiose (39), which argues against the hypothesis that the glycosylation sites in the extracellular loop are sufficient for channel activation. Moreover, the TIP peptide associates with an as yet unidentified site proximal to the C-terminal domain of recombinant, and thus non-glycosylated, ENaC-\(\alpha\) (29).

We mapped crucial residues mediating the binding of TIP peptide to the second transmembrane domain of ENaC-\(\alpha\), focusing on residues Val\(^{607}\), Glu\(^{568}\), and Glu\(^{571}\) (NCBI reference sequence NP_001029.1, amiloride-sensitive sodium channel subunit \(\alpha\) isoform 1, *Homo sapiens*), which were proposed as interaction sites for the TIP peptide in molecular modeling studies using the structure of ASIC1 as a template (10, 29). We generated a 165-residue-long recombinant C-terminal domain of ENaC-\(\alpha\), comprising residues 505–669, from either wild type, single mutant (1M, E571A), double mutant (2M, V567A/E568A), or triple mutant (3M, V567A/E568A/E571A) by performing Ala mutations (Fig. 1A). The WT and 3M mutant ENaC-\(\alpha\) was cloned into a pGEX-4T-3 vector to express recombinant GST-labeled proteins in bacteria. As shown in Fig. 1B, an immunoblot of the purified GST-labeled recombinant protein from BL21 *Escherichia coli* cells detected two main bands at 45 and 26 kDa, corresponding to a GST-labeled ENaC-\(\alpha\) fragment including parts of the palm domain, wrist
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domain, second transmembrane spanning domain, cytoplasmic C terminus, and free GST, respectively.

As shown in Fig. 2, binding of biotinylated TIP peptide to the GST-coupled ENaC-α C-terminal fragment was abrogated when using purified 3M subunit, suggesting that the three residues identified in the molecular docking study (Val567, Glu568, and Glu571) were involved in binding of the TIP peptide to recombinant ENaC-α. Table 1 shows the actual fluorescence intensities of the streptavidin Alexa Fluor 594 bound to biotinylated TIP peptide and eventually with Alexa 549 streptavidin conjugate. Fluorescence of eluted complexes was measured and blanked against naked GST. The data (means ± S.E., n = 4) were analyzed using unpaired t test with Welch’s correction.

Subsequently, we overexpressed full-length WT, single mutant (1M), double mutant (2M), or triple mutant (3M) ENaC-α in HEK293 cells, which do not express significant levels of ENaC-α. Using biotinylated TIP peptide as bait, we pulled down overexpressed 1M, 2M, 3M, or WT ENaC-α from cell lysates and immunoblotted for ENaC-α (Fig. 3A). The left panel of Fig. 3A shows that the TIP peptide pulled down significantly less 3M and 2M ENaC-α, as compared with either WT or 1M ENaC-α subunit (samples normalized for protein concentration). Because the overexpressed 1M and 2M ENaC-α subunits were FLAG-tagged, we also immunoblotted the eluents of the TIP peptide pulldown experiment using the anti-FLAG antibody. Because the TIP peptide mimics the lectin-like domain of TNF, we subsequently performed pulldown studies with biotinylated TNF as bait. As demonstrated in Fig. 3B (right panel), significantly less 3M mutant was pulled down with TNF, as compared with WT ENaC-α subunit. Because there was still significant binding of the TIP peptide and of TNF to the mutant 3M subunit, we hypothesize that this is at least partially mediated by the association with the still intact glycosylated extracellular loop domain. We observed that N-glycosidase F-mediated deglycosylation of WT ENaC-α reduced binding of biotinylated TNF to approximately the same extent as what was found to bind to the 3M subunit. There was also a tendency of reduced binding of TNF upon deglycosylation of the 3M ENaC-α, although it was not significant (Fig. 4).

We subsequently investigated whether mutation of the three residues implicated in the binding of the TIP peptide to the cytoplasmic domain of ENaC-α modifies the gating or open probability of the channel. Because complex formation of ENaC with PIP2 and MARCKS was demonstrated to positively affect its open probability (19), we first investigated whether TIP peptide has the capacity to increase this interaction in H441 cells either overexpressing WT or 3M ENaC-α. To that purpose, we performed immunoprecipitation experiments, using a MARCKS antibody for IP and an ENaC-α antibody for immunoblotting, as described (19). As shown in Fig. 5 (A and B), TIP peptide increased the association between MARCKS and ENaC-α in H441 cells overexpressing WT but not the 3M ENaC-α subunit. TIP peptide also failed to increase the association of MARCKS and ENaC-γ in cells overexpressing the WT or 3M ENaC-α (Fig. 5C).

After transiently transfecting H441 cells with pIRES2-EGFP construct, containing cDNA for either WT or 3M ENaC-α (Fig. 6A), we measured ENaC open probability in single channel patch clamp experiments in H441 cells overexpressing WT, 1M, 2M, or 3M ENaC-α before and 15 min after adding TIP peptide (50 μg/ml). There was no difference in the number of channels between WT, 1M, 2M, or 3M ENaC-α-overexpressing cells in the basal state (WT (n = 9): n = 1.333 ± 0.263; 1M (n = 8): n = 1.250 ± 0.164; 2M (n = 7): n = 1.143 ± 0.143 3M (n = 8): n = 1.375 ± 0.263 (means ± S.E.); NS), 15 min of TIP peptide treatment (50 μg/ml) induced a significant increase in Po in cells overexpressing the WT α subunit (Po before treatment: 0.06 ± 0.022; Po after: treatment 0.2 ± 0.034; means ± S.E.; p < 0.00003) and 1M ENaC-α subunit (Po before treatment: 0.098 ± 0.0179; Po after: treatment 0.172 ± 0.036; means ± S.E.; p = 0.0103), whereas there was no significant increase in Po upon peptide treatment in cells overexpressing 2M (before treatment 0.215 ± 0.0369; after treatment 0.214 ± 0.0381; means ± S.E. p > 0.4) or 3M (before treatment 0.15 ± 0.046; after treatment 0.123 ± 0.032; means ± S.E. p > 0.4). The results from Figs. 5 and 6 demonstrate that residues Val567 and Glu568 located in the interface between the second transmembrane and the C-terminal domains.

FIGURE 1. A, protein sequence of the C-terminal domain of ENaC-α, transcript variant 1 (NM_001038.5), which was used for designing the primers for mutating the proposed binding sites to the lectin-like domain of TNF. B, representative Western blot of the bacterially expressed and purified recombinant TM2-C-terminal domain of WT and 3M ENaC-α. Purified proteins were dialyzed to remove the elution buffer (reduced glutathione), and samples were probed with anti-GST antibody. The arrow is pointing to the recombinant GST-ENaC-α TM2-C-terminal domain fusion protein.

FIGURE 2. GST pulldown experiment. Measurement of binding of biotinylated TIP peptide to WT and 3M recombinant TM2-C-terminal domain of GST-ENaC-α. WT or 3M GST-ENaC-α decorated glutathione-Sepharose 4B beads were incubated with biotin-labeled TIP peptide and eventually with Alexa 549 streptavidin conjugate. Fluorescence of eluted complexes was measured and blanked against naked GST. The data (means ± S.E., n = 4) were analyzed using unpaired t test with Welch’s correction.

FIGURE 3. A, GST pulldown experiment with biotinylated TIP peptide. B, GST pulldown experiment with biotinylated TIP peptide and TNF. C, GST pulldown experiment with biotinylated TIP peptide and TNF after deglycosylation.
Glu568 in the second transmembrane domain of ENaC-α are crucial for the ENaC-MARCKS association and for the activation of open probability by the TIP peptide. Moreover, as demonstrated in Fig. 6A, a whole cell voltage clamped patch clamp experiment in HEK 293 cells overexpressing 3M ENaC-α supported these data, because the TIP peptide no longer activated Na+ uptake in these cells, in contrast to its effects in cells overexpressing the WT subunit (data not shown and Ref. 38). As shown in Fig. 7, overexpression of 3M ENaC-α in H441 cells also impairs activation of amiloride-sensitive Na+ uptake by hTNF (100 ng/ml), in contrast to cells overexpressing the WT subunit.

Val567, Glu568, and Glu571 Are Not Involved in TIP Peptide-mediated Inhibition of ENaC-α Ubiquitination—ENaC activity is determined not only by its open probability time but also by the expression of functional channels at the plasma membrane, the latter of which is at least partially regulated by Nedd4-2-mediated ubiquitylation and subsequent degradation (12). We therefore assessed whether mutation of residues Val567, Glu568, and Glu571 to Ala affects the increase in ENaC-α expression in H441 cells treated with the TIP peptide (29). H441 cells were permanently transfected with PiggyBac dual promoter vector for integration of wild type or 3M ENaC-α into the target genome. Positive cells were selected by puromycin treatment in the media and could be detected by RFP (Fig. 8A). As shown in Fig. 8B, TIP peptide treatment decreased pulldown of ubiquitinated ENaC-α from H441 cell lysates in the presence of the proteasome inhibitor bortezomib. This experiment was performed using agarose beads coupled with tandem ubiquitin-binding entities 2 (TUBE2) (40), which bind equally to both Lys53 and Lys48 ubiquitin chains, the latter of which are involved in proteasomal degradation of ENaC. The inhibitory effect of the TIP peptide on ENaC-α ubiquitination occurred to the same extent in H441 cells overexpressing wild type or 3M ENaC-α subunit. In the presence of 125 ng/ml of pneumolysin (PLY), a pneumococcal pore-forming toxin that was shown to reduce ENaC expression in H441 cells (29), more ubiquitinated ENaC-α was pulled down with TUBE2-coupled agarose beads in both wild type and 3M overexpressing H441 cells, and in both cases the TIP peptide blunted this increase (Fig. 8C).

Taken together, these data indicate that residues Val567, Glu568, and Glu571 in the TM2 domain of ENaC-α are not involved in effects of the TIP peptide on ENaC-α ubiquitination and degradation in the absence or presence of the bacterial toxin PLY. These results support our previous hypothesis that the interaction between the lectin-like domain of TNF with glycosylated
residues in the extracellular loop of ENaC-α mediates its effects on ENaC expression (38).

Effects of V567A, E568A, and E571A Mutations on ENaC Subunit Association—Because ENaC functions most efficiently as a complex of α, β, and γ subunits, we investigated whether 3M ENaC-α can still efficiently associate with the β and γ subunits either in a cell-free system or at the cellular plasma membrane. Recombinant GST-coupled full-length 3M ENaC-α and WT ENaC-α, β, and γ fusion proteins were used to perform GST pulldown assays. WT GST-ENaC-α and 3M GST-ENaC-α fusion proteins were immobilized to glutathione-Sepharose before adding thrombin-cleaved ENaC-β or ENaC-γ proteins. The eluted proteins were subjected to SDS-PAGE and Western blotting to examine binding between the ENaC subunits. As shown in Fig. 9, there is direct binding between GST-ENaC-α, ENaC-β (Fig. 9A) and ENaC-γ (Fig. 9B), which occurred with both WT and 3M GST-ENaC-α constructs. However, when using the 3M GST-ENaC-α fusion protein, the interactions with the β and γ subunits were attenuated, as compared with the WT subunit. Upon co-transfection of HEK293 cells with either WT GFP-ENaC-α, FLAG-ENaC-β, and FLAG-ENaC-γ or 3M GFP-ENaC-α, FLAG-ENaC-β, and FLAG-ENaC-γ, the cells were surface biotinylated and lysed, and ENaC complexes were immunoprecipitated using ENaC-α antibody. Eluted complexes were further separated on agarose-avidin beads to obtain the biotinylated, membrane-expressed ENaC heterotrimers. The eluted complexes were then probed for ENaC-β and ENaC-γ and FLAG (Fig. 9D). Fig. 9C shows the transfection efficiency of the FLAG-tagged constructs. Despite displaying reduced binding to the other subunits in a cell-free system, these results thus indicate that the 3M ENaC-α subunit still efficiently associates with the β and γ subunits at the plasma membrane of HEK 293 cells, which is also substantiated by the similar number of channels in H441 cells either overexpressing WT or 3M ENaC-α.

Discussion

Increased generation of pro-inflammatory cytokines represents a first-line defense mechanism against bacterial infections of the lung. TNF represents a prominent member among the pro-inflammatory mediators, as evidenced by the occurrence of spontaneous tuberculosis in patients chronically treated with TNF inhibitors for rheumatoid arthritis (41). Although increased TNF generation promotes bacterial destruction, it can also reduce the transcriptional expression of all three ENaC subunits and the protein expression of ENaC-α, in a TNF receptor-dependent manner, which impairs alveolar liquid clearance (ALC), for which ENaC is crucial (33, 35–37). TNF moreover reduces capillary endothelial barrier function (42). Impaired ALC in combination with capillary leak can as such give rise to the generation of potentially lethal pulmonary edema. In most cases this does not happen, indicating that protective mechanisms exist during inflammation that can preserve or restore ENaC activity and ALC capacity. As such, catecholamines, in a cAMP-dependent manner, were shown to markedly up-regulate ALC, an effect that is mediated by both ENaC and cystic fibrosis transmembrane conductance regulator (43). However, these mechanisms can be impaired in the presence of excessive levels of the pro-inflammatory chemokine IL-8 or TGF-β1 (44, 45). Moreover, TGF-β was demonstrated to reduce expression of the β-subunit of ENaC (32).
This therefore stresses the need to identify alternative mechanisms that can restore ENaC function during inflammation.

We have recently demonstrated that, in sharp contrast to the TNF receptor 1 binding site, the lectin-like domain of TNF, mimicked by the TIP peptide (also called AP301 and solnatide), activates ENaC activity in the presence or absence of increased pulmonary TNF concentrations or bacterial toxins, such as pneumococcal-derived PLY (29, 34). The TIP peptide was recently shown to significantly improve lung fluid clearance in a phase IIa clinical trial in patients with acute lung injury (ClinicalTrials.gov identifier NCT01627613).

ENaC activation by the TIP peptide occurs through a direct mechanism and possibly involves two distinct binding sites for the TIP peptide in ENaC-α. The first one consists of glycosylated Asn residues in the extracellular loop of the subunit (38), whereas modeling studies suggested that the second site is located in the TM2 domain in the vicinity of the cytoplasmic C-terminal region (29). The aims of this study were to identify the intracellular binding domain of ENaC-α and to define which of these sites (extracellular versus cytoplasmic) regulates open probability time and membrane expression of the channel, both of which define ENaC activity (1, 6).

Our results indicate that residues Val567 and/or Glu568 play an important role in the binding of the TIP peptide to a recombinant domain at the interface of TM2 and the C terminus of ENaC-α and to define which of these sites (extracellular versus cytoplasmic) regulates open probability time and membrane expression of the channel, both of which define ENaC activity (1, 6).

FIGURE 6. A, representative bright field and immunofluorescent pictures of H441 cells transfected with either WT or 3M ENaC-α GFP plasmids. B, whole cell patch clamp experiment assessing the activation of ENaC channel in the 3M ENaC-α overexpressing HEK293 cells in the absence or presence of the TIP peptide and amiloride. C, representative current traces of the single channel patch clamp experiment using the above-transfected H441 cells. D–G, single channel patch clamp experiments (n = 7–9, means ± S.D.) TIP peptide-mediated increase in Na⁺ uptake in WT and 1M ENaC-α overexpressing H441 cells. This effect is blunted in 2M and 3M ENaC-α-overexpressing H441 cells.
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FIGURE 7. Effect of hTNF (100 ng/ml) on amiloride-sensitive Na⁺ uptake in H441 cells overexpressing wild type ENaC-α (A) or 3M mutant ENaC-α (B) (n = 3; means ± S.D.). CTRL, control.

FIGURE 8. A, representative bright field and immunofluorescent pictures of H441 cells transfected with either WT or 3M ENaC-α PiggyBac transposon RFP plasmids upon puromycin selection. B, effect of TIP peptide (50 μg/ml) on polyubiquitination of WT and 3M ENaC-α. The polyubiquitinated proteins were pulled down with agarose-TUBE2 beads and probed for ENaC-α or ubiquitin. C, representative Western blot assessing the effect of PLY (125 ng/ml) on the polyubiquitination of WT and 3M ENaC-α, upon pretreatment or not with TIP peptide. The data (means ± S.E., n = 3) were analyzed using unpaired Student t test. IB, immunoblot; BZM, bortezomib.
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involved in regulating the open probability of the channel (19).
As a consequence, the TIP peptide no longer increased ENaC open probability in H441 cells overexpressing the 3M mutant subunit, whereas it did so in cells overexpressing WT ENaC-α. In view of the reduced capacity of 3M ENaC-α to associate with the other 2 subunits in a cell-free system, one could potentially argue that the TIP peptide fails to activate ENaC in cells overexpressing the 3M mutant ENaC-α, because they no longer make functional channels. However, this is unlikely because basal currents and channel density were identical between H441 cells overexpressing wild type or 3M ENaC-α, and 3M ENaC-α stably associated with the other two subunits at the plasma membrane of HEK 293 cells. Taken together, these results suggest that the mutant subunit can still make functional heterotrimeric channels. It is also unlikely that there are still sufficient native channels present in the 3M ENaC-α overexpressing H441 cells, because otherwise these channels should have been activated by the TIP peptide. In view of the impaired capacity of 3M ENaC-α to bind to TIP peptide; 2) To respond to TIP peptide-mediated stimulation of MARCKS interaction; and 3) to increase open probability of ENaC upon TIP peptide treatment, we propose that residues Val567 and Glu568 in the TM2 domain of ENaC-α are crucially involved in the activation of ENaC by the lectin-like domain of TNF.

Apart from its open probability and maturation, ENaC activity is also defined by its expression level at the plasma membrane. The number of functional channels in the apical membrane depends upon a balance between the insertion rate of the channel and the rate of removal from the apical membrane (2). In both transfected and untransfected epithelial cells, the rate of ENaC degradation is controlled by the rate of Nedd4-2-mediated ENaC ubiquitination. Binding of Nedd4-2 to ENaC is regulated by phosphorylation (12). Our results demonstrate that TIP peptide reduced ubiquitination of ENaC-α in H441 cells, even in the presence of the bacterial toxin PLY, the main virulence factor of Streptococcus pneumoniae, which was shown to reduce ENaC expression (30). In contrast to the results regarding open probability, TIP peptide was still able to reduce ENaC-α ubiquitination in H441 cells overexpressing the 3M mutant subunit, even in the presence of PLY. This result indicates that the two identified residues in the TM2-C-terminal domain interface of ENaC-α are not involved in the effects of the peptide on ENaC degradation and expression. This hypothesis is strengthened by our recent observations in HEK 293 cells indicating a crucial role for N-glycosylation in the activating effect of the TIP peptide on ENaC expression (38).

Because the TIP peptide can potentially bind to two spatially distinct sites in ENaC-α—the extracellular loop cellular loop and the interface between TM2 and the C-terminal domain—it is unclear at this point how the peptide reaches the intracellular side of the channel. The distance between the extracellular loop binding site and the more intracellular TM2-C-terminal domain is too big for the peptide to be able to reach both of them at the same time. However, cellular N-deglycosylation was shown to be a crucial prerequisite for the subsequent activation of the channel by the TIP peptide (29, 38). The possibility that the mere binding of hTNF or of the TIP peptide to glycosylated Asn residues in the extracellular loop of ENaC-α is sufficient to activate the channel is unlikely. Indeed, a mutant TIP peptide, which was shown to exhibit nearly identical binding

![Figure 9](image-url)
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capacity to N,N'-diacyctethylchitobiose as the wild type TIP peptide (39) failed to activate ENaC (29). This substantiates our hypothesis that a second site within ENaC-α is required for the actual activation of the channel by the TIP peptide or TNF.

Our previously published findings indicated that the TIP peptide no longer activated ENaC in H441 cells in the presence of β-methyl-cyclohextrin, an inhibitor of caveolae-mediated internalization (29). Because binding to N-glycosylated residues was shown to affect internalization and expression of ion channels, including ENaC-α (46–48), we propose that the TIP peptide is internalized together with ENaC upon binding to glycosylated Asn residues in the extracellular loop and as such can reach the more intracellular binding site in the TM2 of the subunit. Mutation of the identified residues in the TM2 site of ENaC-α also impairs activation of amiloride-sensitive Na⁺ uptake in H441 cells by hTNF. Although these data do not firmly prove that TIP peptide or hTNF bind to the ENaC-α TM2 domain in cells, they do suggest that the identified residues in the TM2 domain are crucial for both TIP peptide and hTNF-induced ENaC activation. Despite the demonstrated binding of hTNF and TIP peptide to the TM2 domain of ENaC-α in a cell-free system, we cannot completely exclude the possibility that this site of ENaC-α is only indirectly involved in the TNF-induced activation of the channel and does not physiologically interact with the cytokine in a cellular context.

ENaC activation by the lectin-like domain of TNF might be physiologically relevant, especially under conditions during which not only TNF but also its soluble receptors are generated. Such a TNF-soluble TNF receptor complex no longer activates the TNF receptors but can still promote lung liquid clearance, as we have shown previously in a flooded rat lung model (34). TNF has been shown to be able to interact with membrane, independent from its receptors, and the cytokine was shown to increase ALC in mice lacking both TNF receptors (49, 50). Knock-in mice expressing a mutant TIP in which the lectin-like domain is dysfunctional have a reduced edema reabsorption capacity and a reduced ENaC-α expression, upon instillation of a low dose of pneumolysin. These observations substantiate a potential physiological role for the lectin-like domain of TNF in ALC (29, 51).

In conclusion, this study has identified a novel site within the interface between TM2 and the C-terminal domains of ENaC-α that mediates activation of the channel by the lectin-like domain of TNF. It remains to be studied whether this site can also be activated by other ligands and how the two potential TNF-mediated activation sites for ENaC-α (N-glycosylation sites in the extracellular loop versus the TM2 domain) can interact with one another.

Experimental Procedures

Antibodies and Reagents—Uncomplexed and biotinylated TIP peptide was synthesized and purified by AmbioPharm, Inc. (North Augusta, SC). Biotin-labeled TNF was purchased from ACROBiosystems. LPS-free PLY was purified from a recombinant Listeria innocua 6a strain, expressing PLY as described previously (30). The batch of PLY used in this study had a specific activity of 1.25 × 10⁷ hemolytic units/mg. ENaC-α 59 and ENaC-β 60 antibodies (19), as well as the FLAG-tagged ENaC-β and ENaC-γ plasmids, were generated by D. C. E. ENaC-α (H-95) was purchased from Santa Cruz (sc-21012). ENaC-γ antibody was from Abcam (ab3468). PR-619 (DUBs inhibitor), 1,10-Phenantroline (DUBs, JAMM inhibitor) and agarose-TUBE2 was from LifeSensors, Inc. Anti-MARCKS, anti-K48 polyubiquitin, HRP-conjugated anti-biotin (DS57; 5571), anti-FLAG (9A3; 8146), and the HRP-conjugated goat anti-rabbit (7074), as well as goat anti-mouse (7076) antibodies, were purchased from Cell Signaling Technology, Inc. Dynabeads streptavidin M280, protein G, and Lipofectamine 3000 were from Invitrogen.

Cells—Clara cell-like H441 lung adenocarcinoma cells were obtained from ATCC and were grown in RPMI 1640 medium supplemented with 2 mm l-glutamine, 1.5 g/liter sodium bicarbonate, 4.5 g/liter glucose, 10 mm HEPES (pH 7.4), 10% fetal bovine serum, and 1% penicillin/streptomycin (v/v). Human embryonic kidney cells (HEK 293) were used for overexpressing WT and triple mutant ENaC-α. These cells were cultured in a DMEM culture medium containing 4.5 g/liter glucose, l-glutamine, 10% fetal bovine serum, and 1% penicillin/streptomycin (v/v) at 37 °C and 5% CO₂.

Site-directed Mutagenesis—Human sodium channel non-voltage-gated 1α transcript variant 1 plasmid was purchased from OriGene (Rockville, MD). This was used as a template for site-directed mutagenesis. Point mutations were introduced into cDNA encoding α-subunit with GeneArt® site-directed mutagenesis PLUS system (Life Technologies). Mutagenic primers were designed individually with the Primer Design Program provided on the Manufacturer’s website and ordered from Integrated DNA Technologies (Corvalle, IA). Sequences were as follows: single mutant (1M, E571A) forward: 5'-GGTGGAGATGCTGCTGCTCTTTGACC-3' and reverse: 5'-GGTCAAAAGACGAGGCGCAGCCATCTCCACC-3'; double mutant (2M, V567A/E568A) forward: 5'-CGGTGTTTGTCTGTGTCGGCGGATGGCTAGCTGCT-3' and reverse: 5'-ACGAGTCGATCGCTATCGCGCGCAACAACACC'-3'; and triple mutant (3M, V567A/E568A/E571A) forward: 5'-GGTCAAAAGACGAGGCGCAGCCATCTCCACC-3' and reverse: 5'-GTTGTTTGTCTGCTGCTGCTTCGTTGAC-3'. Point mutations were introduced that changed the codon for two Glu (at positions 568 and 571) and Val (at position 567) in the ENaC-α isoform 1 reference sequence to that for Ala, thus resulting in replacement of these residues by Ala in the reference ENaC-α subunit at the corresponding binding sites of TNF, as suggested by molecular docking studies using ASIC1 as a template (29). Whereas replacement of Glu with Ala is non-conservative and can as such affect conformation of the subunit, substitution of Val with Ala is conservative and should have minimal disruptive effect on the physical and chemical properties of the protein. Mutant strands were synthesized by PCR with Accuprime Pfx-based DNA polymerase using 30 ng of WT ENaC-α cDNA. The recombination step was carried out according to the manufacturer's protocol and proceeded immediately to transformation. Aliquots of OneShot® MAX Efficiency® DH5α™-T1® competent cells were incubated with the amplified DNA for 15 min on ice. After 30 s of heat shock at 42 °C and 2 min on ice, the cells were grown in SOC medium for 1 h at 37 °C with shaking
at 225 rpm. The cells were spread on LB-agar (20 g of LB broth and 15 g of agar, with ddH₂O added to a final volume of 1 liter, then autoclaved) plates containing the appropriate antibiotic (ampicillin) for the plasmid vector and incubated overnight (16–18 h) at 37 °C. Single colonies were picked and cultured in LB broth (20 g of LB broth with ddH₂O added to a final volume of 1 liter, then autoclaved) containing the appropriate antibiotic overnight (16–18 h) at 37 °C with shaking at 225 rpm. The DNA was extracted with the Qiagen plasmid miniprep kit. The DNA concentration was measured with NanoDrop ND-1000 spectrophotometer by 3-fold determinations. All constructs were checked by restriction site mapping and sequencing. The extracted DNA was sent to ACGT, Inc. (Wheeling, IL) for sequencing. The sequencing result was evaluated with the ExPaSy translate tool. The wild type and triple mutant ENaC-α cDNAs were subcloned into three different vectors: pGEX-4T-3 for preparing GST-tagged bacterially expressed recombinant protein, pIRE2-EGFP for transiently transfecting H441 cells with GFP plasmid, and PiggyBac dual promoter (RFP/puromycin) vector for integrating the WT and triple mutant ENaC-α into the target genome. Aliquots of Mix & Go competent cells (Strain Zymo 5α) were incubated with the ENaC cDNAs for 5 min on ice. The cells were spread on LB-agar (20 g of LB broth and 15 g of agar with ddH₂O added to a final volume of 1 liter, then autoclaved) plates containing the appropriate antibiotic (Ampicillin) for the plasmid vector and incubated overnight (16–18 h) at 37 °C.

PiggyBac Integration into H441 or HEK293 Cells—The cells were grown to 60–80% confluence and transfected with the mixture of transposone vector clone, transposase vector, and transfection reagent in serum-free medium, following the manufacturer’s guidelines. After 72 h, when the transposon vector was integrated into the genome of the cells, the puromycin selection was applied to the cells (5 μg/ml). The cells were pelleted, and the supernatant containing the GST cleaved proteins was harvested. The cells efficiently transposed were puromycin-resistant and RFP-positive.

ENaC-α Transient Transfection of H441 or HEK293 Cells—70–80% confluent cells were transfected with the pIRE2-EGFP constructs of WT and 3M ENaC-α using X-tremeGENE HP transfection reagent (Roche) according to the manufacturer’s protocol, using a 2:1 ratio. Positive cells were detected by green fluorescent staining. After 48 h, the cells were collected, lysed, and either tested by SDS-PAGE or used in different assays.

Immunoprecipitation—ENaC-α-transfected human lung adenocarcinoma epithelial cells grown in 60-mm culture flasks were washed with 1× PBS, scraped, and lysed in 400 μl of 20 mM Tris-HCl, pH 7.4 buffer containing 0.15 M NaCl, 1% Nonidet P-40, 2 mM EDTA, and protease inhibitors. The lysates (corresponding to 0.5 mg of total protein) were incubated with 0.1 μmol of biotinylated TIP peptide or TNF, coupled to MyOne™ streptavidin M280 magnetic beads (corresponding to ~100 μl of Dynabeads slurry) overnight at 4 °C. Afterward, the beads were washed three times with 1× PBS (2% BSA) and eluted in 150 μl of Laemmli buffer, upon which supernatants were analyzed by Western blotting.

Deglycosylation of ENaC-α—ENaC-α-overexpressing H441 cells were grown in 100-mm culture dishes, washed with 1× PBS, scraped, and lysed in 600 μl of 20 mM Tris-HCl, pH 7.4 buffer containing 0.15 M NaCl, 1% Nonidet P-40, 2 mM EDTA, and protease inhibitors. The lysates were centrifuged 10,000 × g for 10 min, and the supernatants were incubated with 10 units of peptide N-glycosidase F from *E. coli* at 37 °C for 2 h.

GST Pulldown Assay—Biotinylated TIP peptide, biotinylated rhTNF (0.5 nmol of peptide/recombinant protein) were incubated with 0.3 nmol each of GST, GST-wild type, and GST-3M C-terminal domain of ENaC-α fusion proteins, prepared as described (52), coupled to glutathione-Sepharose beads (corresponding to 1/10 of Microspin column, GE Healthcare, ~60 μl of glutathione-Sepharose slurry) for 1 h at 4 °C. Afterward, the beads were washed and incubated with streptavidin Alexa Fluor® 594 conjugate for another 1 h at room temperature in dark. The complex was eluted in 200 μl of 10 mM glutathione elution buffer. Each eluent was transferred into a 96-well plate, and the absorbance was measured in triplicates at 600-nm wavelength against blank.

Subunit Association Studies—GST-ENaC-α wild type, GST-ENaC-α triple mutant, GST-ENaC-β, and GST-ENaC-γ fusion proteins were expressed and purified as previously described (52, 53). In separate tubes, 40 μg of each fusion protein was incubated with 50 μl of prewashed glutathione-Sepharose 4B beads and 100 μl of PBS at 4 °C for 4 h with end over end mixing. GST-ENaC-β and GST-ENaC-γ fusions proteins bound to glutathione-Sepharose 4B beads were subjected to thrombin cleavage by incubating them with 1 unit of thrombin in thrombin cleavage buffer (20 mM Tris-HCl, pH 8.5, 0.5 mM CaCl₂, 1 mM DTT, 100 mM NaCl, and 0.1% Tween X-100) at 4 °C for 2 h with end over end mixing. The glutathione-Sepharose 4B beads were pelleted, and the supernatant containing the GST cleaved ENaC-β or ENaC-γ fusion protein was collected and incubated with the wild type GST-ENaC-α or 3M GST-ENaC-α fusions proteins immobilized to glutathione-Sepharose 4B beads at 4 °C for 6 h with end over end mixing. The complexes were washed three times with PBS before being resuspended in 60 μl of 2× Laemmli sample buffer and boiled for 10 min. Eluted proteins were subjected to SDS-PAGE, Western blotting with anti-ENaC-β 60 (19) and anti-ENaC-γ 2102 (54) polyclonal antibodies, and chemiluminescence detection (SuperSignal Dura).

In Vitro Agarose TUBE2 Pulldown assay—Untreated or treated (50 μg/ml TIP peptide, 125 ng/ml PLY) WT or 3M ENaC-α overexpressing HEK293/H441 cells in the presence of proteasome inhibitor (100 nM bortezomib) were washed and lysed with Nonidet P-40 lysis buffer (20 mM Tris-HCl, pH 7.4 buffer containing 0.15 M NaCl, 1% Nonidet P-40, 2 mM EDTA, protease inhibitors, and Ub/Ubl proteases inhibitor 50 μM PR619, inhibitor of metalloproteases 5 mM o-phenanthroline). The input sample was removed from the lysates for analysis by Western blotting. The remaining lysates (~400 μl of lysis buffer containing 1–2 μg of total protein) were added to 20 μl of 50% slurry of agarose-TUBE2 and incubated for 3 h at 4 °C on a rocker platform. Afterward, the beads were washed and eluted in Laemmli buffer. Each eluent was probed for ENaC-α and polyubiquitin.

Whole Cell and Single-channel Patch Clamp Studies—Whole cell voltage-clamped patch clamp experiments in HEK293 and
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H441 cells overexpressing either WT or 3M ENaC-α were performed as described previously (38). Single channel patch clamp experiments in H441 cells were performed at room temperature using the cell-attached patch configuration as described (29). Patch pipette and extracellular bath solutions consisted of a physiological H441 cell saline containing the following: 95 mM NaCl, 3.4 mM KCl, 0.8 mM CaCl₂, 0.8 mM MgCl₂, and 10 mM HEPES or 10 mM Tris, titrated with 0.1 N NaOH or HCl to a pH of 7.3–7.4. The TIP peptide (50 μg/ml) or hTNF (100 ng/ml) were added to H441 cells or to HEK 293 cells cultured on glutaraldehyde-fixed, collagen-coated Millipore-CM filters (Millipore, Billerica, MA) attached to the bottoms of small Lucite rings. The open probability (Pₒ) of a single channel was calculated by dividing NPₒ by the number of channels in a patch. For our experiments, we determined mean Pₒ for 10 min before addition of the TIP peptide and for 20 min after addition.

Surface Biotinylation/Immunoprecipitation—WT or 3M ENaC-α GFP constructs were co-transfected with FLAG-tagged ENaC-β and γ-plasmids in HEK293 cells. The cells were washed twice with ice-cold PBS and biontinylated using Pierce cell surface protein isolation kit (Thermo Scientific) according to the manufacturer’s instructions. The cells were then trypsinized and collected by centrifugation (500 × g for 3 min). After lysing the cells with the provided lysis buffer, the samples were subjected to immunoprecipitation. The clarified supernatants were incubated with 2 μg of ENaC-α antibody for 2 h at 4 °C on a head over head shaker and then added to the equilibrated protein G magnetic beads (Invitrogen) to further incubate overnight at 4 °C on a head over head shaker. The protein G complexes were washed and disrupted with 1% of SDS in lysis buffer for 80 min at 37 °C. The beads were then separated on a magnet, and the supernatant was used for isolation of biotinylated proteins. The biotinylated proteins were eluted with hot Laemmli buffer containing 50 mM DTT. Eluted proteins were subjected to SDS-PAGE and Western blotting with anti-ENaC-β antibody (40) polyclonal antibodies.


References


