The Lectin-like Domain of TNF Increases ENaC Open Probability through a Novel Site at the Interface between the Second Transmembrane and C-terminal Domains of the -Subunit

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Journal Title: Journal of Biological Chemistry  
Volume: Volume 291, Number 45  
Publisher: American Society for Biochemistry and Molecular Biology | 2016-11-04, Pages 23440-23451  
Type of Work: Article | Final Publisher PDF  
Publisher DOI: 10.1074/jbc.M116.718163  
Permanent URL: https://pid.emory.edu/ark:/25593/s6cq7

Final published version: http://dx.doi.org/10.1074/jbc.M116.718163

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Accessed January 16, 2020 4:54 AM EST
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Received for publication, January 27, 2016, and in revised form, September 15, 2016 Published, JBC Papers in Press, September 19, 2016, DOI 10.1074/jbc.M116.718163


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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-\( \alpha \), 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-\( \alpha \), as a critical site for binding of the TIP peptide. We generated Ala replacement mutants in this region of ENaC-\( \alpha \) and examined its interaction with TIP peptide (3M, V567A/E568A/E571A; 2M, V567A/E568A; and 1M, E571A). 3M and 2M ENaC-\( \alpha \), but not 1M ENaC-\( \alpha \), displayed significantly reduced binding capacity to TIP peptide and to TNF. When overexpressed in H441 cells, 3M mutant ENaC-\( \alpha \) formed functional channels with similar gating and density characteristics as the WT subunit and efficiently associated with the \( \beta \) and \( \gamma \) subunits in the plasma membrane. We subsequently assayed for increased open probability time and membrane expression, both of which define ENaC activity, following addition of TIP peptide. TIP peptide increased open probability time in H441 cells overexpressing wild type and 1M ENaC-\( \alpha \) channels, but not 3M or 2M ENaC-\( \alpha \) channels. On the other hand, TIP peptide-mediated reduction in ENaC ubiquitination was similar in cells overexpressing either WT or 3M ENaC-\( \alpha \) subunits. In summary, this study has identified a novel site in ENaC-\( \alpha \) 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: \( \alpha \), \( \beta \), and \( \gamma \), the former of which is crucial to obtain functional channels (2). A fourth unique subunit, ENaC-\( \delta \), can also form channels with the \( \beta \) and \( \gamma \) subunits but exhibits biophysical and pharmacological properties that are divergent from those of \( \alpha \) 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, \( P_o \) (8). A variety of external factors, including acidic pH, Na\(^+\), and proteases modify ENaC activity through interaction with domains

\(^6\)The abbreviations used are: ENaC, epithelial sodium channel; TM, transmembrane; ASIC1, acid-sensing ion channel 1; PIP\(_2\), phosphatidylinositol bisphosphate; MARCKS, myristoylated alanine-rich C kinase substrate; ARDS, acute respiratory distress syndrome; TUBE2, tandem ubiquitin binding entities 2; P2Y, phospholipase; ALC, alveolar liquid clearance; ddH\(_2\)O, double distilled H\(_2\)O; RFP, red fluorescent protein; GFP, green fluorescent protein.
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-α, β, and γ transcription, as well as ENaC-α 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-α subunit (29, 38). Intriguingly, our recent experimental and molecular modeling data indicated that two distinct binding sites exist for the TIP peptide in ENaC-α. The first one, obtained through mutational analysis in a heterologous cell system, consists of glycosylated Asn residues (Asn	extsuperscript{272}, Asn	extsuperscript{293}, Asn	extsuperscript{312}, Asn	extsuperscript{397}, and Asn	extsuperscript{511}) 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-α 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-α Binding to the TIP Peptide—**

We 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 αAsn	extsuperscript{511} being crucial (29, 38). This suggests that glycosylated residues in the extracellular loop of ENaC-α 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-α (29).

We mapped crucial residues mediating the binding of TIP peptide to the second transmembrane domain of ENaC-α, focusing on residues Val	extsuperscript{267}, Glu	extsuperscript{568} and Glu	extsuperscript{571} (NCBI reference sequence NP_001029.1, amiloride-sensitive sodium channel subunit α isofrom 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-α, 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-α 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-α fragment including parts of the palm domain, wrist
A Novel Activation Site in ENaC-α

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 implicated in the lectin-like domain of TNF, with a Glycine-rich motif, were not critical for binding. These results are consistent with previous studies showing that the lectin-like domain of TNF associates with two different domains in ENaC-α: a glycosylated domain in the extracellular loop (38), as well as a site including residues Val567 and Glu568, located in the interface between the second transmembrane and the C-terminal domains.

We subsequently investigated whether mutation of the three residues implicated in the binding of the TIP peptide to the cytoplasmic domain of ENaC-α modulates the gating or open probability of the channel. Because complex formation of ENaC with PIP2 and MARCKS was demonstrated to positively affect channel gating, 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 measurements 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 P_o in cells overexpressing the WT α subunit (P_o before treatment: 0.06 ± 0.022; P_o after treatment: 0.2 ± 0.034; means ± S.E.; p < 0.00003) and 1M ENaC-α subunit (P_o before treatment: 0.098 ± 0.0179; P_o after treatment: 0.172 ± 0.036; means ± S.E.; p = 0.0103), whereas there was no significant increase in P_o 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 of the C-terminal domain of ENaC-α are critical for the interaction with MARCKS and, by association, with the channel. Furthermore, the increase in P_o upon TIP peptide treatment in cells overexpressing the WT and 1M ENaC-α subunits suggests that the TIP peptide has the capacity to increase this interaction in H441 cells overexpressing WT, 1M, 2M, or 3M ENaC-α.
Glu\textsuperscript{568} in the second transmembrane domain of ENaC-\(\alpha\) 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-\(\alpha\) supported these data, because the TIP peptide no longer activated Na\textsuperscript{+} 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-\(\alpha\) in H441 cells also impairs activation of amiloride-sensitive Na\textsuperscript{+} uptake by hTNF (100 ng/ml), in contrast to cells overexpressing the WT subunit.

Val\textsuperscript{567}, Glu\textsuperscript{568}, and Glu\textsuperscript{571} \textit{Are Not Involved in TIP Peptide-mediated Inhibition of ENaC-\(\alpha\) 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-ubiquitination—ENaC-\(\alpha\) subunit. In the presence or absence of peptide N-glycosidase. The cell lysates were deglycosylated and then immediately added to the biotinylated TNF-decorated beads. B, densitometry of the immune-reactive bands corresponding to ENaC-\(\alpha\), pulldown experiment of FLAG-tagged 1M and 2M ENaC-\(\alpha\) with TIP peptide. The data (means ± S.E., \(n = 3\)) were analyzed using unpaired test with Welch’s correction. IB, immunoblot; PD, pull down.

the media and could be detected by RFP (Fig. 8A). As shown in Fig. 8B, TIP peptide treatment decreased pulldown of ubiquitinated ENaC-\(\alpha\)-ENaC-\(\alpha\) 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 Lys\textsuperscript{53} and Lys\textsuperscript{48} ubiquitin chains, the latter of which are involved in proteasomal degradation of ENaC. The inhibitory effect of the TIP peptide on ENaC-\(\alpha\)-ubiquitination occurred to the same extent in H441 cells overexpressing wild type or 3M ENaC-\(\alpha\) 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-\(\alpha\) 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 Val\textsuperscript{567}, Glu\textsuperscript{568}, and Glu\textsuperscript{571} in the TM2 domain of ENaC-\(\alpha\) are not involved in effects of the TIP peptide on ENaC-\(\alpha\)-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, cactecholamines, 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. Indeed, mutation of these residues to Ala significantly reduced the stimulatory effect of the TIP peptide on complex formation between ENaC and MARCKS, a protein shown to be...
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.
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 still efficiently 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 heterotrimers. 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 ENα-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 ENα expression (30). In contrast to the results regarding open probability, TIP peptide was still able to reduce ENα-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 ENα-α are not involved in the effects of the peptide on ENα 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 ENα expression (38).

Because the TIP peptide can potentially bind to two spatially distinct sites in ENα-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 ENα-α is sufficient to activate the channel is unlikely. Indeed, a mutant TIP peptide, which was shown to exhibit nearly identical binding

FIGURE 9. GST pulldown assays assessing direct binding between ENα subunits. WT GST-ENα-α, 3M GST-ENα-α, WT GST-ENα-β, and WT GST-ENα-γ fusion proteins were expressed and purified from bacterial cells. WT GST-ENα-α and 3M GST-ENα-α immobilized to glutathione-Sepharose were incubated with thrombin cleaved GST-ENα-β and GST-ENα-γ fusion proteins. A and B, the eluted complexes were resolved on 4 – 20% SDS-PAGE gels, and the blots were probed for ENα-β (A) and ENα-γ (B). C, HEK cells were co-transfected with either WT ENα-α, -β, and -γ subunits or 3M ENα-α subunit and subjected to surface biotinylation. The cells were lysed, and ENα complexes were immunoprecipitated with ENα-α antibody. Eluted complexes were further separated on agarose-avidin beads to obtain ENα heterotrimers expressed in the plasma membrane. D, eluted complexes were then probed for ENα-β, ENα-γ, and FLAG, because β and γ constructs were FLAG-tagged. The representative WB was obtained from three independent experiments. IB, immunoblot; IP, immunoprecipitation.
A Novel Activation Site in ENaC-α

capacity to N,N'-diacetylchitobiose 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-cyclodextrin, 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 TNF in which the lectin-like domain of TNF is only indirectly involved in the TNF-induced activation of the channel and does not physiologically interact with the cytokine in a cellular context.

In conclusion, this study has identified a novel site within the 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 (D5A7; 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 Lipo-pectamine 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'-GGTGGCGGCGATGGCTGAGCTCGT-3' and reverse: 5'-GGTCAAAAGACAGCCACGCATCCATCACC-3'; double mutant (2M, V567A/E568A) forward: 5'-CGGTGTTTGCT-TGTGGCCGCGATGCTGTAGCTGT-3' and reverse: 5'-ACGACGTCAACCCATCGCGCCACAGAACAACCC-3'; and triple mutant (3M, V567A/E568A/E571A) forward: 5'-GGTCAAAAGACAGCCACGCATCCATCACC-3' and reverse: 5'-GGTGTGTGTGTGTGTGCAGCG-ATGGCTGCGCTGTCTTGCAC-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.
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 Elizabethkingia miricola 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 lyases 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 1 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...
A Novel Activation Site in ENaC-α

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 NP 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 bisulfitylated 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 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. After washing the columns three times with wash buffer, the biotinylated proteins were eluted with hot Laemmli buffer containing 50 µM DTT. Eluted proteins were subjected to SDS-PAGE and Western blotting with anti-ENaC-β 60 (19) and anti-ENaC-γ (54) polyclonal antibodies.


References
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