Epithelial Inducible Nitric-oxide Synthase Is an Apical EBP50-binding Protein That Directs Vectorial Nitric Oxide Output*

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Nitríc oxide (NO), produced via inducible NO synthase (iNOS), can modulate polarized epithelial processes such as solute transport. Given the high reactivity of NO, we hypothesized that optimal NO regulation of polarized epithelial functions is achieved through compartmentalization of iNOS, allowing local NO delivery to its molecular targets. Here, we show that iNOS localizes to the apical domain of epithelial cells within a submembranous protein complex tightly bound to cortical actin. We further show that iNOS can bind to the apical PDZ protein, EBP50 (ezrin-radixin-moesin-binding phosphoprotein 50), an interaction that is dependent on the last three COOH-terminal amino acids of iNOS, SAL, but requires the presence of additional unknown cellular proteins. Mutation of these three COOH-terminal residues abolishes the iNOS-EBP50 interaction and disrupts the apical association of iNOS in transfected cells, showing that this COOH-terminal motif is essential for the correct localization of iNOS in epithelial cells. Apically localized iNOS directs vectorial NO production at the apical proximal tubule epithelial cell surface. These studies define human epithelial iNOS as an apical EBP50-binding protein and suggest that the physical association of iNOS with EBP50 might allow precise NO modulation of EBP50-associated protein functions.

Inducible nitric-oxide synthase (iNOS) produces high concentrations of the ubiquitous signaling molecule, NO. The role of high NO output from iNOS in epithelial cells is unclear, although there is increasing evidence that epithelial NO exerts its effects on polarized cell functions. For example, NO modulates the activity of the cystic fibrosis transmembrane regulator (CFTR) and the apical renal proximal tubule epithelial cell (PTEC) Na⁺/H⁺ exchanger (NHE3). NO is highly diffusable and reactive within cells and with neighboring cells and molecules. We therefore hypothesized that endogenous NO-dependent regulation of apical epithelial functions, such as solute transport, might optimally be achieved through localization of iNOS to the apical cell compartment, allowing local NO delivery to its molecular targets.

How might iNOS be targeted to the apical cell compartment? Recent studies have shown that PDZ domain-containing proteins play an important role in trafficking, sorting, and retention of several apical proteins. PDZ domains mediate protein-protein interactions via binding to specific amino acid sequences at the COOH terminus of target proteins. One such protein, ezrin-radixin-moesin-binding phosphoprotein 50 (EBP50), contains two PDZ domains that recognize the COOH-terminal sequence (ST/TL) (9). EBP50 is highly abundant within apical epithelial microvilli (10) and retains several apical proteins via PDZ domain-mediated interactions, including NHE3 and CFTR (11, 12). EBP50 also exerts regulatory effects on its binding partners; for example, EBP50 confers cyclic AMP-mediated regulation of NHE3 (11). Inspection of the last three COOH-terminal residues of human iNOS shows that they contain a potential PDZ domain-binding motif, SAL (13). One possibility may be, therefore, that iNOS could interact with a PDZ domain via its three COOH-terminal amino acids.

In this study, we show for the first time that iNOS localizes to the apical domain of polarized epithelial cells in association with the cortical actin cytoskeleton. Further, we show that iNOS can bind to the PDZ protein EBP50 via its three COOH-terminal amino acids. These residues are also found to be essential for the correct apical localization of iNOS within epithelial cells. We demonstrate that iNOS produces NO in a vectorial manner predominantly at the apical cell surface. This work defines epithelial iNOS as a polarized apical protein and provides a mechanism whereby locally delivered NO may influence polarized epithelial cell functions.

**EXPERIMENTAL PROCEDURES**

**Materials**—iNOS, EBP50, E-cadherin, and caveolin-1 monoclonal antibodies were from Transduction Laboratories. The mAb to β1-integrin was from Chemicon International. Zona occludens-1 (ZO-1) polyclonal antibody was from Zymed Laboratories Inc. EBP50 anti-sera was a gift from Prof. A. Bretscher (Cornell University, Ithaca, NY), described in Ref. 10. iNOS polyclonal Ig was made as described (14) and also was obtained from Transduction Laboratories. Biotinylated secondary antibodies, fluorescein avidin, and Vectashield were from Vector Laboratories. Cy3-conjugated anti-rabbit Ig was from Jackson ImmunoResearch Laboratories. Interleukin 1α, tumor necrosis factor-α, and interferon-γ were from Peprotech. pGEX-6P-1 was from Amersham Biosciences. Unless stated otherwise, all other chemicals were from Sigma-Aldrich.

**Cell Culture and Transfection**—Primary human PTEC cultures were established in serum-free medium as described (15). Human bronchial epithelial 16HBE14o− cells (a gift from Dr. D. Gruenert, University of California, San Francisco), grown in RPMI 1640/10% FCS, were stably transfected with human iNOS cDNA (a gift from Dr. I. Charles, Wellcome Laboratories) cloned into the XhoI sites of the expression vector.
pC neo (Promega) using Perfectipid 6 (Invitrogen). iNOS-expressing and control cells were cloned by limiting dilution in the presence of G418. iNOS expression in 50% of the cells was achieved after 48 h of incubation with 5 μM n-butyl acrylamide. A similar approach was used to derive stable iNOS transfectants in MDCK II cells (a kind gift from R. Mostov, University of California). For each construct ≥ 5 independent clones were analyzed with identical results. Cells were polarized by growth on a semipermeable support with separate apical and basolateral chambers (Anapore filters, Nunc, Abingdon, UK).

**NO Production**—NO was measured as nitrite + nitrate (NOx) using the Griess assay following reduction of nitrate to nitrite (16). Vectorial NO production was measured by the hemoglobin method (17). iNOS activity was assayed by arginine to citrulline conversion (NOSdetec™, Stratagene). NO production was inhibited by the iNOS inhibitor L-NIL (L-nitroarginine methyl ester) (Calbiochem).

**Immunoblotting**—Cells grown on Anopore filters (Nalgene Nunc) were immunostained as described (14). iNOS was visualized using the appropriate biotinylated secondary antibody (1 h) and fluorescein avidin (1 h) (both diluted 1:200). ZO-1 and EBP50 were visualized using Cy3-conjugated secondary antibody (1:500). Cells were mounted in Vectashield® and analyzed by confocal microscopy (Bio-Rad MRC 1024 running LaserSharp software). iNOS-expressing cells were quantified as the percentage of the area of the epithelial sheet in a given micrograph using NIH Image software, version 1.62, to measure the areas of stained cells with pixel intensities above the threshold level.

**Apical iNOS Staining** was quantified by determining the mean fluorescence intensities of apical and cytoplasmic areas of the cell and then subtracting the background value measured over the nucleus. The product of the mean fluorescent intensity and the measured area was used to determine the relative amounts of iNOS in each compartment.

**Cell Fractionation**—All protocols were performed at 4°C. PTEC were washed three times in PBS, scraped into lysis buffer (50 mM Tris, pH 7.5, 0.3 μM aprotinin, 2 μM leupeptin, 3 μM pepstatin, 1 mM phenylmethylsulfonyl fluoride), and lysed by Dounce homogenization and sonication. After the nuclei were pelleted at 10,000 x g for 15 min, the remaining low speed supernatant (LSS) was centrifuged at 200,000 x g for 1 h to yield a high speed supernatant (HSS) (cytosol) and pellet (HSP) (particulate fraction). Fractions analyzed for iNOS by Western blotting were developed using ECL and quantified by digital densitometry.

**Sucrose Equilibrium Density Centrifugation**—The HSP was resuspended in 1 mM lysis buffer and overlaid onto a 20–55% linear sucrose gradient (11.5 ml) over a 0.5 ml 64% sucrose cushion. Following centrifugation at 200,000 x g for 16 h at 4°C, fractions (0.7 ml) were collected from the bottom of the gradient and analyzed by immunoblotting.

**Membrane Extractions**—The HSP was resuspended in either 0.25 or 1.0 M KCl, 0.1 M Na2CO3, 0.1 M NaOH, 0.1% Triton X-100, 1% Triton X-100 + 250 mM NaCl, or 0.1% SDS in lysis buffer. Membranes were extracted at 0°C for 10 min (Na2CO3, and NaOH) or 60 min (remander) and centrifuged at 200,000 x g for 1 h, and supernatants and pellets were analyzed by immunoblotting.

**Glutathione S-transferase (GST) Fusion Constructs**—The 10 COOH-terminal residues of human iNOS (sequence data available from GenBank™/EMBL/DDBJ under accession no. X73029) were cloned in frame into the expression vector pcDNAHisMaxTA (Invitrogen, Paisley, UK). This construct was transfected into HEK293 cells using LipofectAMINE 2000 (Invitrogen), and the protein was purified from cell lysates 24 h later using immobilized metal affinity chromatography. The protein was 95% pure as judged by Coomassie staining of protein run on a SDS-polyacrylamide gel.

**Results**

**iNOS Localization to the Apical Domain of Pioneered Epithelial Cells**—We first established optimal conditions for iNOS induction in human PTEC. Stimulation of PTEC with 10 ng/ml interleukin 1α, 200 units/ml interferon-γ, and 10 ng/ml tumor necrosis factor-α for 24 h produced maximal NO release compared with unstimulated cells (128.14 ± 13.2 nmol/million cells 24 h −versus 19.75 ± 10.96, p < 0.01, n = 3). Where indicated, this cytokine mixture was used in all experiments. PTEC NO synthase activity was calcium-independent in total cell lysates and increased significantly after cytokine treatment compared with unstimulated cells (66.08 ± 0.9 pmol/mg versus 0.025 ± 0.002 pmol/mg respectively, p < 0.05, n = 2). iNOS induction was confirmed by identification of a 135-kDa protein in cytokine-treated PTEC by Western blotting. These results showed that NO production from cytokine-stimulated PTEC was iNOS-dependent. In addition, there is constitutive iNOS activity in unstimulated cells.

The subcellular distribution of iNOS was examined by immunocytochemistry using two different iNOS antibodies, and identical results were obtained with each antibody. iNOS staining was not detected in unstimulated PTEC. Following cytokine treatment, iNOS was induced in a proportion of PTEC (median value of 7.75% iNOS positive cells/high power field in eight separate experiments). Confocal microscopy showed that iNOS localized to the apical domain (Fig. 1A, green staining, panels 1, 4, and 7) above the tight junction marker ZO-1 (Fig. 1A, red staining, panel 2), which delineates the transition between the apical and basolateral domains of a polarized epithelial cell. Note in these cells that ZO-1 staining is confined to a limited area at the junction between cells that does not extend to the basal surface of the cells (indicated by the dotted line in Fig. 1A, panels 1–3). In addition, iNOS was found to colocalize with the apical marker EB50 (Fig. 1A, panels 4–9). This pattern of apical staining was observed in all iNOS-positive cells analyzed in polarized PTEC monolayers (from eight kidney preparations). The distribution of iNOS within PTEC, quantified by image analysis, showed that 73.4 ± 4.22% of iNOS was located in the apical compartment.

The apical localization of iNOS is also seen in epithelial cells stably transfected with iNOS expression vectors. These were derived for MDCK cells (Fig. 1B) and the respiratory epithelial line 16HBE14o– (Fig. 1C). In all cases, iNOS localized to the apical compartment (left-hand column, green staining). Cells were additionally stained for markers of epithelial polarity (middle column), which were E-cadherin (basolateral marker, Fig. 1B, panel 2, and C, panels 2 and 8) and β1-integrin (Fig. 1B, panel 5, and C, panel 5). On prolonged culture of polarized 16HBE14o– cells, some areas showed stacking of cells to two cells deep. In these areas, iNOS was also localized to the apical surface of the cells (Fig. 1C, panels 7–9).

Given the limits of resolution of confocal microscopy (pixel size 0.172 μm), we were unable to conclude whether iNOS was directly associated with the plasma membrane in the apical compartment or was localized to an adjacent submembranous region. We therefore adopted a biochemical approach to explore further the association of iNOS with the plasma membrane. Biochemical analysis of lysates extracted from cytokine-treated PTEC (Fig. 2A) demonstrated that iNOS is present in both apical and basolateral fractions (Fig. 2B).
cytosolic (HSS, Fig. 2A) and particulate (HSP, Fig. 2A) cell fractions. Digital densitometry showed that 29.33 ± 2.06% (n = 4) of total cellular iNOS was recovered from the HSS, a similar proportion to that found for the actin-associated protein EBP50 (data not shown). iNOS enzyme activity was present in both HSS (8.17 ± 0.28 pmol/mg min⁻¹) and HSP (1.03 ± 0.05 pmol/mg min⁻¹), with the latter representing 11.2% of total cellular iNOS activity. The percentage of iNOS in the particulate fraction assayed by immunoblotting is about half that determined by confocal microscopy; this may reflect some loss of iNOS from this compartment on cell lysis and centrifugation. The lower iNOS activity in the particulate fraction may reflect poor substrate availability. However, these data confirm that a significant proportion of total cell iNOS is compartmentalized and enzymatically active within a membrane-rich, particulate fraction.

We further analyzed the characteristics of iNOS within the particulate fraction by resuspending the HSP in lysis buffer and then subjecting it to fractionation by sucrose equilibrium density centrifugation. iNOS sedimented in dense fractions (Fig. 2B, lanes 13–17), similar to the distribution of actin and the majority of the actin-associated membrane protein E-cadherin (lanes 8–17). iNOS was distinct from caveolin-1, a non-actin-associated plasma membrane protein and marker of caveolae (light fractions, Fig. 2B, lanes 3–6). Membrane vesicles containing E-cadherin were also found in light fractions (Fig. 2B, lanes 3 and 4) but were distinct from iNOS.

This analysis of the material within the HSP shows that biochemical fractionation under these conditions does not provide a complete separation into distinct subcellular compartments. However, taken together, these data indicate that iNOS within the particulate fraction is not within a light lipid-rich structure but rather may be present in a large submembranous protein complex associated with the cortical cytoskeleton. To explore further the nature of this association, we extracted the HSP with a series of salt and detergent solutions (Fig. 2C). We found that buffers containing high salt concentrations (up to 1.0 M KCl) were ineffective in extracting iNOS (Fig. 2C, lane 4). However, iNOS was completely extracted by 0.1 M NaOH (Fig. 2C, lane 5), confirming that it is not an integral membrane protein. The protein remained insoluble in the presence of non-ionic detergents (0.1% and 1% Triton X-100; Fig. 2C, lanes 6 and 7, respectively), and 0.1% SDS (Fig. 2C, lane 9) but was entirely solubilized by 1% Triton X-100 + 250 mM salt (Fig. 2C, lane 8) and 1% SDS (Fig. 2C, lane 10). These data indicate a tight interaction with the particulate fraction although not as tight as for actin-binding proteins such as supervillin (18).

Given the striking apical localization of iNOS and the biochemical data suggesting that iNOS is associated with the cytoskeleton, we examined the relationship between iNOS and cortical actin. Confocal microscopy demonstrated that iNOS (Fig. 2, D and J) colocalized with cortical actin (Fig. 2, E and K) in polarized PTEC (yellow pixels in Fig. 2, F and L). Furthermore, iNOS was redistributed into the cytosol following actin disruption by cytochalasin D (Fig. 2, G–I), confirming that iNOS is associated with cortical actin either via a direct or indirect interaction.

**iNOS Interacts with EBP50**—Given its potential COOH-terminal PDZ binding motif (SAL), we examined whether iNOS might localize to the apical domain via an interaction with EBP50. We tested this hypothesis using pull-down assays with GST fused to (i) the 10 COOH-terminal residues of iNOS (GST-iNOS) or (ii) a mutated COOH-terminal sequence (GST-iNOSmut) (Fig. 3A). Following incubation of GST-iNOS with 16HBE140− cell lysates, a 50-kDa band and two smaller bands (~45 kDa) were detected in the precipitate as shown on the Coomassie-stained gel in Fig. 3B. These bands were not detected when 16HBE140− lysates were incubated with GST only. Western blot analysis of the same precipitates showed that the 50-kDa band corresponded to human EBP50 (Fig. 3C). The two lower molecular weight bands were also recognized by the anti-EBP50 mAb, suggesting that they might represent EBP50 fragments. In contrast, EBP50 was not present in the precipitates of 16HBE140− cell lysates incubated with GST-iNOSmut (Fig. 3C). Pull-down assays using PTEC lysates showed an identical specific interaction between EBP50 and GST-iNOSmut but no binding of EBP50 to GST-iNOSmut or GST alone (Fig. 3D). Thus, the COOH-terminal tail of human
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FIG. 2. iNOS is present in a particulate fraction tightly bound to cortical actin. A, Western blotting of cytokine-stimulated PTEC fractions. Lane 1, total cell lysate (50% cell equivalent of lanes 2–4). Nuclei were pelleted at 10,000 × g (low speed pellet (LSP)). The remaining LSS was centrifuged at 200,000 × g to yield cytosolic (HSS, lane 3) and particulate fractions (HSP, lane 4). B, sucrose equilibrium density centrifugation of the HSP. 17 gradient fractions were collected and analyzed by immunoblotting using anti-iNOS mAb (1:500), anti-E-cadherin mAb (1:1000), anti-actin mAb (1:1000), and anti-caveolin-1 mAb (1:500). C, membranes (HSP) were extracted with lysis buffer alone (lane 1) or with buffer containing 0.25 M Na2CO3 (lane 2), 1% Triton X-100 (lane 3), or 0.1% SDS (lane 4). Lane 10, membranes extracted with 1% SDS at 70 °C for 10 min (positive control). For each condition, high speed supernatants (S) and pellets (P) were analyzed by immunoblotting using anti-iNOS mAb. B and C were performed on two different cell preparations. D–I, confocal micrographs of cytokine-stimulated PTEC stained using anti-iNOS mAb (D and G) and TRITC-phalloidin (1:100) (E and H). F, merged image of D and E; I, merged image of G and H. G and H, iNOS and actin staining after preincubation with 10 μM cytochalasin D. J–L, confocal images of transverse sections of cytokine-stimulated PTEC stained for iNOS (J) and actin (K), and the merged image is shown (L). Bar, 10 μm.

FIG. 3. Interactions of iNOS with EBP50. A, sequences of the wild type (GST-iNOSwt) and mutated (GST-iNOSmut) GST-iNOS COOH terminus fusion proteins. B–D, pull-down assays using ~2 μg of each fusion protein. B, GST only or GST-iNOSwt incubated with 16HBE14o− cell lysates (from ~5 × 106 cells/lane). Bound proteins were visualized by Coomassie staining. C and D, GST only, GST-iNOSwt or GST-iNOSmut incubated with 16HBE14o− cells (from ~5 × 106 cells/lane) or PTEC (D, from ~5 × 106 cells/lane) cell lysates. Bound proteins were analyzed by immunoblotting using anti-EBP50 mAb (1:1000). Experiments were performed on three different cell preparations. E, interaction of purified recombinant EBP50 expressed in HEK cells with GST-iNOS COOH-terminal fusion proteins. Bound material obtained at varying concentrations of EBP50 (indicated in nM) with GST-iNOSmut (lane B) or GST-iNOSwt (lanes C–G) is shown, with EBP50 detected as described above. Input material (21.4 ng) corresponding to the total amount of recombinant EBP50 in the 10 nM incubation is run in lane A. Molecular mass markers are indicated in kDa to the left of each panel.

iNOS binds to EBP50, an interaction that is dependent on the three COOH-terminal amino acids, SAL. It is also possible that iNOS may interact with other members of the EBP50 family, such as E3KARP, which migrates with a similar mobility on SDS-polyacrylamide gels. However, E3KARP has a different tissue distribution than EBP50 and is not found within the proximal tubule cells used in the above pull-down experiments (19).

As shown in Fig. 1, iNOS and EBP50 colocalize within epithelial cells at the apical compartment of the cell. We attempted to demonstrate an interaction within cells by co-immunoprecipitation of iNOS and EBP50 from epithelial cells. However, we were unable to do this either from cytokine-treated PTECs or iNOS-transfected cells. Expression of iNOS even in stably transfected cells was only found in about 10–20% of the cell population, and following the incubations required for immunoprecipitation, a significant amount of iNOS degraded to lower molecular weight peptides (data not shown). Both of these factors may limit the ability to detect iNOS/
EBP50 interactions in a cellular context. Because the pull-down experiments were performed in whole lysates, they do not differentiate between a direct or indirect interaction of iNOS with EBP50. To address this problem directly, we purified full-length recombinant EBP50 expressed in human HEK293 cells. Using this material, we were unable to demonstrate a direct interaction between EBP50 and iNOS (Fig. 3E) despite a very sensitive Western blot assay system that could easily detect nanomole amounts of EBP50. This suggests that any interaction of iNOS and EBP50 must require the presence of additional proteins found in whole cell lysates, as used for the pull-down experiments (Fig. 3, A–D).

Does the potential interaction of the COOH terminus of iNOS with the PDZ domain-containing protein EBP50 play a role in localizing iNOS to the apical domain? To test for this possibility directly, we purified full-length recombinant EBP50 expressed in human HEK293 cells. Using this material, we were unable to demonstrate a direct interaction between EBP50 and iNOS (Fig. 3E) despite a very sensitive Western blot assay system that could easily detect nanomole amounts of EBP50. This suggests that any interaction of iNOS and EBP50 must require the presence of additional proteins found in whole cell lysates, as used for the pull-down experiments (Fig. 3, A–D).

Fig. 4. Apical localization of iNOS is dependent on its COOH-terminal PDZ-binding domain. A, merged confocal xz image of MDCK cells stably transfected with full-length INOSwt or INOSmut bearing the same COOH-terminal mutation as described in the legend to Fig. 3. Cells were stained with antibodies to iNOS (green) and ZO-1 (red) as before. The dotted line indicates the plane of the supporting membrane on which the cells were grown. B, quantification of the % of iNOS localized to the apical compartment in MCK transfectants as indicated. *, indicates a significant difference between the transfectants (p = 0.00002, t test, n = 12).

Localization of iNOS in the apical compartment of epithelial cells. Taken together with the other data presented above, a plausible explanation for this would be an interaction of iNOS with EBP50 leading to its apical distribution and association with the cortical actin cytoskeleton.

iNOS Directs Vectorial NO Production—To determine whether iNOS delivers NO locally at the apical domain, we measured apical and basal NO output from cytokine-treated PTEC monolayers. Measurement of NO in these two compartments showed rapid NO release in the apical chamber with no significant output basally (Fig. 5). Apical t-NIL-sensitive NO concentration reached 6.82 ± 0.9 μM in the period 12–18 h after cytokine addition, compared with the basal NO output of 0.09 ± 0.09 μM (P < 0.005, n = 4, Fig. 5). These data show that apical compartmentalization of iNOS results in vectorial NO release at the apical PTEC surface.

DISCUSSION

We describe here the novel observation that iNOS in epithelia is localized to the apical compartment and directs synthesis of NO in a vectorial fashion at the apical epithelial surface. We demonstrate that iNOS is tightly associated with cortical actin and has a COOH-terminal PDZ-binding domain that can interact with the apical submembranous scaffold protein, EBP50. This domain is essential for the correct localization of iNOS to the apical compartment. These findings show that iNOS, in common with the other NOS isoforms, endothelial NOS (20) and neuronal NOS (21), is targeted to a specific cellular location, suggesting that subcellular compartmentalization of NO synthesis is important in limiting NO production to the region of the cell where required. Given the high reactivity of NO, it would be advantageous to the cell to locate the site of high concentration NO synthesis in close proximity to its site of production.
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action, limiting access to other cell compartments. This would maximize the efficiency of NO delivery to its targets such as apical transporters and reduce potential toxicity elsewhere in the cell. One interesting possibility is that NO may modulate the function of other proteins complexed with EBP50, such as the apical NHE3 or the c-yes-associated protein, YAP65.

The imaging results show that iNOS is localized in close proximity to the apical membrane of both cytokine-treated and iNOS-transfected epithelial cells (Fig. 1). Because iNOS will only be produced in the human kidney following stimulus with pro-inflammatory cytokines, we have not been able to study the distribution of iNOS in vivo, as renal biopsy material is not collected from patients with, for example, severe sepsis in which iNOS might be expected to be induced. However, given the range of different epithelial cell types in which we observed this localization, we suggest that this is a general feature of iNOS within epithelia. Confocal imaging cannot differentiate the localization of iNOS either to within the plasma membrane or immediately subjacent to it. Biochemical analysis of the distribution of iNOS within epithelial cells (Fig. 2) shows that it is tightly bound to a particulate cell fraction with properties that suggest close association with the actin cytoskeleton. This is supported by the co-localization of iNOS and cortical actin that is disrupted by the actin depolymerizing agent, cytochalasin (Fig. 2, D-I). We found a similar association of iNOS and cortical actin within murine macrophages (22), suggesting that a similar association occurs in these cells.

We show that the COOH terminus of iNOS can mediate an interaction with the PDZ protein EBP50 (Fig. 3). This is an important molecule that has a number of different binding partners. Through its interaction with ezrin, it is anchored to the actin cytoskeleton (10). In turn, it binds to the apical ion channel NHE3 (23), the CFTR (12, 25), the c-yes-associated protein YAP65 (26), the β2 adrenergic receptor (27), the P2Y1 purinergic receptor (9), the platelet-derived growth factor receptor (28), G protein-coupled receptor kinase A (29), Trp4 and Trp5 (30), phospholipase C (30), EP164, nadrin, calpain-like protease (31), the membrane adaptor protein Cbp (32), and the β₁ subunit of the H⁺-ATPase (33). These interactions have important functional consequences, for example in modulating NHE3 activity (23) and regulating the chloride conductance of the CFTR by promoting interaction between two CFTR molecules (34). The structural basis for these interactions, in common with all PDZ-containing proteins, is though binding to the COOH-terminal three amino acids of the target protein. The COOH terminus of human iNOS is MSAL, which is a good consensus for interaction with the PDZ domains of EBP50 (the COOH-terminal sequences of all EBP50-interacting proteins are summarized in Ref. 31). A similar COOH-terminal consensus sequence, ATRL, is also found in murine iNOS (35).

This COOH-terminal sequence is essential for in vitro binding of iNOS to EBP50 (Fig. 3) and for in vivo apical localization (Fig. 4). Surprisingly, isolated pure EBP50 was not able to bind to this sequence on its own (Fig. 3), suggesting that any in vivo interaction requires additional stabilizing factors. One of these might be posttranslational modifications of the COOH terminus of iNOS modulating binding to PDZ domains, as has been shown for the COOH terminus of the β2 adrenergic receptor (36) and the tumor suppressor PTEN (24). In addition, the possibility for interactions between multiprotein complexes held together by EBP50 is very great, and the relative affinity of these interactions may depend on the presence of other proteins bound within the complex.

The requirement for the three COOH-terminal amino acids of iNOS for efficient apical localization to the apical domain suggests that the interaction of iNOS with EBP50 is important in vivo. A number of mechanisms might account for this polarized distribution depending on the iNOS PDZ-biding domain. First, iNOS may be sorted to both apical and basolateral destinations but is retained in the apical compartment by binding to EBP50. Second, the COOH-terminal domain may interact with a PDZ domain within the Golgi, to facilitate anterograde transport to the apical compartment. This might be EBP50, or possibly another PDZ domain-containing protein such as CAL, which is localized within the Golgi and has been shown to bind to CFTR and modulate its surface expression. Third, the COOH-terminal PDZ-interacting domain may itself be a sorting determinant to direct iNOS to the apical compartment. It is difficult to distinguish between these possibilities. We have observed that in subconfluent cells, iNOS is localized within an uncharacterized vesicle population within the cytoplasm. In some cells this is concentrated into the Golgi region, suggesting that traffic through the Golgi could determine subcellular localization of iNOS. However, mutation of the three terminal amino acids of iNOS do not seem to alter the localization of iNOS within this vesicle population, although the precise effects on Golgi localization have not been determined. Intriguingly, wild type iNOS is also localized to the tips of long filopodia in subconfluent cytokine-treated human PTECs. The exact molecular mechanism involved in the apical targeting of iNOS within epithelial cells remains unknown, other than the fact that it is dependent on the COOH-terminal three amino acids of iNOS.

In conclusion, we have shown that human epithelial iNOS localizes to the apical domain of polarized cells, tightly bound to cortical actin. Further, we show that iNOS binds to the apical PDZ protein EBP50 via its three COOH-terminal amino acids, SAL. Apical localization of iNOS within cells requires the three COOH-terminal amino acids, showing the importance of this interaction in vivo. iNOS directs vectorial NO production at the apical cell surface. This work has important implications for the identification of novel roles for NO in the regulation of apical epithelial cell functions. The precise nature of the iNOS-EBP50 interaction and its importance for correct iNOS localization and function within the apical compartment of polarized epithelial cells are yet to be elucidated and will be the subject of future studies.

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