Binding of CAP70 to Inducible Nitric Oxide Synthase and Implications for the Vectorial Release of Nitric Oxide in Polarized Cells

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In this article we analyze the mechanisms by which the C-terminal four amino acids of inducible nitric oxide synthase (NOS2) interact with proteins that contain PDZ (PSD-95/DLG/ZO-1) domains resulting in the translocation of NOS2 to the cellular apical domain. It has been reported that human hepatic NOS2 associates to EBP50, a protein with two PDZ domains present in epithelial cells. We describe herein that NOS2 binds through its four carboxy-terminal residues to CAP70, a protein that contains four PDZ modules that is targeted to apical membranes. Interestingly, this interaction augments both the cytochrome c reductase and $\cdot\text{NO}$ -synthase activities of NOS2. Binding of CAP70 to NOS2 also results in an increase in the population of active NOS2 dimers. In addition, CAP70 participates in the correct subcellular targeting of NOS2 in a process that is also dependent on the acylation state of the N-terminal end of NOS2. Hence, nonpalmitoylated NOS2 is unable to progress toward the apical side of the cell despite its interaction with either EBP50 or CAP70. Likewise, if we abrogate the interaction of NOS2 with either EBP50 or CAP70 by fusing the GFP reporter to the carboxy-terminal end of NOS2 palmitoylation is not sufficient to confer an apical targeting.

INTRODUCTION

The messenger molecule nitric oxide is known to play a key role in the immune system due predominantly to its antimicrobial and antitumor activities. Inducible nitric oxide synthase (NOS2) is generally assumed to be transcriptionally up-regulated as a result of multiple proinflammatory stimuli, and its action is associated to the release of large amounts of nitric oxide (MacMicking *et al.*, 1997). Although it was initially believed that, unlike NOS1 or NOS3, the regulation of the synthesis of nitric oxide by NOS2 was practically nonexistent, more recent data seem to indicate that NOS2 activity is exquisitely regulated within mammalian cells. In this regard, NOS2 is known to interact with at least 10 different proteins (calmodulin, caveolin-1, -2, and -3, NAP110, kalirin, Rac1, Rac2, α -actinin 4, and EBP50; Daniliuc *et al.*, 2003; Kone *et al.*, 2003 and references therein).

In addition, palmitoylation of Cys3 is necessary for NOS2 activity in both transfected and induced cells (Navarro-Lérida *et al.*, 2004b, 2006). Finally, phosphorylation of NOS2 by src, and probably by other kinases too, also regulates its activity and translocation to detergent-insoluble domains (Hausel *et al.*, 2006).

The inhibitory effect of caveolin binding to NOS2 was first demonstrated when peptides derived from the scaffolding domain of both caveolin-1 and -3 were able to completely abrogate NOS2 activity (García-Cardena *et al.*, 1997). However, when NOS2 is induced in C2C12 mouse myotubes with a mixture of cytokines, it associates with caveolin-1 and only marginally with caveolin-2 and -3 (Navarro-Lérida *et al.*, 2004a). A yeast two-hybrid screen using the first 70 amino acids of NOS2 as bait revealed that the N-terminal end of the protein associates with NAP110 and kalirin, two proteins that promote the monomerization of NOS2 and inhibit its activity (Ratovitski *et al.*, 1999a,b). Conversely, the stable overexpression of Rac2 in Raw 264.7 cells augmented LPS-induced nitrite generation without measurably affecting NOS2 protein abundance (Kunciewicz *et al.*, 2001).

In addition, we have recently showed that NOS2 becomes palmitoylated at position 3 of its sequence (Navarro-Lérida *et al.*, 2004b, 2006). In fact, the nonpalmitoylated Cys3Ser mutant accumulated in perinuclear areas of the cell and was unable to progress along the sorting routes. In fact, palmitoylation was necessary for NOS2 in order to reach full activity. We also demonstrated that both cytokine-induced and transfected NOS2 became palmitoylated, a modification that was necessary for full activity.

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Abbreviations used: CAP70, CFTR-associated protein of 70 kDa; CFTR, cystic fibrosis transmembrane conductance; EBP50, ezrin-radixin-moesin-binding phosphoprotein of 50 kDa; GFP, green fluorescent protein; hNOS2, human hepatic NOS2; mNOS2, mouse macrophage NOS2; NOS, nitric oxide synthase; NOS2, inducible nitric oxide synthase; PDZ, PSD-95/DLG/ZO-1.

In this article we have focused on the subcellular localization of NOS2 mediated by its carboxy-terminal amino acids, which are known to display a type I consensus binding sequence toward proteins containing PDZ (PSD-95/DLG/ZO-1) domains (Hung and Sheng, 2002). This motif comprises residues falling within the sequence: -X-(S/T)-X-(V/L)-COOH. Interestingly, because of a poorly understood splicing process, the final amino acids of human heart NOS2 are E-P-K-G-T-R-L-COOH (Adams *et al.*, 1998), which differ from those found in human hepatocytes and chondrocytes: L-E-M-S-A-L-COOH (Charles *et al.*, 1993; Geller *et al.*, 1993). NOS2 from murine tissues display less heterogeneity in this region, ranging from -P-K-G-T-R-L-COOH in the case of rat hepatocytes to -P-K-A-T-R-L-COOH in mouse macrophages (Wood *et al.*, 1993). In any case, all these sequences fall into the type I motif for PDZ domain-interacting proteins, although this variability might well indicate that different subsets of proteins with PDZ domains might specifically interact with NOS2 in certain tissues, hence determining a particular subcellular localization. For instance, in human epithelial cells, transfected human hepatic NOS2 (hNOS2) interacts with EBP50, a protein that localizes to the apical membrane of polarized cells, and this interaction selectively drives $\cdot$ NO production toward the apical side of the monolayer (Glynn *et al.*, 2002).

In this article we describe how NOS2 becomes selectively targeted to the apical membrane in multiple tissues. By means of a yeast two-hybrid assay we have established that in human heart, the carboxy-terminal end of NOS2 interacts with EBP50 as well and does so with higher affinity than its hepatic counterpart. In addition, we have discovered the interaction of NOS2 with CAP70, a protein that possesses four PDZ domains and is very abundantly expressed in kidney, liver, small intestine, and certain epithelia (Wang *et al.*, 2000). To our surprise, binding of purified recombinant EBP50 and CAP70 to two different recombinant isoforms of NOS2 increases significantly the $\cdot$ NO-synthesizing activity and cytochrome c reductase of the latter. This might be indicative that, as in the case of NOS3, the carboxy-terminal stretch of amino acids might regulate the activity of the enzyme limiting the flow of electrons from the reductase domain of the protein toward the heme-oxygenase domain.

MATERIALS AND METHODS

Materials

Glutamine, antibiotics, cell culture media (Dulbecco's modified Eagle's medium), sulfanilic acid, *N*-(1-naphthyl)-ethylenediamine dihydrochloride, cytochrome-c, oxy-ferrous hemoglobin, nickel-nitrilotriacetic acid resin, transfection reagent Escort-IV, X- β -Gal, cytochalasin-D, oligonucleotides, and Hoechst were purchased from Sigma (St. Louis, MO). Trypsin-EDTA and fetal bovine serum were from BioWhittaker Inc. (Walkersville, MD). The source of the various antibodies used in this work was as follows: rabbit polyclonal anti-CAP70 and anti-GST were raised in our laboratory using recombinant proteins as immunogens; anti- β -catenin and anti-EBP50 (anti-NHERF-1) were purchased from Sigma; the rabbit polyclonal anti-GFP serum was raised in our laboratory using purified recombinant GFP as previously described (Navarro-Lérida *et al.*, 2002). Protein A-Sepharose, 2',5'-ADP-Sepharose, electrochemiluminescence (ECL) reagents, Cy2- and Cy3-labeled secondary antibodies were from Amersham Biosciences (Piscataway, NJ). L-arginine and tetrahydrobiopterin were obtained from Merck (Rahway, NJ). 3-Amino-1,2,4-triazole was from Fluka (Ronkonkoma, NY). The yeast strains, pretransformed Human Heart "Matchmaker" cDNA library and the lithium acetate transformation kit were purchased from BDClontech (San Jose, CA). The Alexa488-labeled phalloidin was purchased from Molecular Probes (Eugene, OR).

Recombinant Expression in *Escherichia coli* and Purification of Proteins

Recombinant expression of mouse macrophage NOS2 (mNOS2) and human hNOS2 was performed in *E. coli* at 23°C using the pCWori plasmid. Purification was performed using a Ni-NTA affinity resin that recognized the

hexa-His tag introduced at the amino-terminal end followed by purification using ADP-Agarose that binds to the carboxy-terminal end of the protein. In both cases, calmodulin was coexpressed with NOS2 in order to achieve a proper final folding of NOS2 as described (Rodríguez-Crespo and Ortiz de Montellano, 1996; Gerber *et al.*, 1997; Nishida and Ortiz de Montellano, 1999). Cloning of EBP50 and CAP70 was performed in frame with glutathione S-transferase (GST) in the pGEX2-T vector (Amersham Pharmacia Biotech) and purification was performed using glutathione-agarose following the instructions of the manufacturer.

Cell Culture and Transfection, Immunoprecipitation, and Immunoblotting

These cell biology techniques were performed using standard procedures as described in Navarro-Lérida *et al.* (2002, 2004a,b).

Determination of $\cdot$ NO Release and Cytochrome c Reduction

Because nitric oxide decomposes in nitrites and nitrates, we determined the concentration of nitrites in the samples using the Griess assay. A volume of 0.5 ml of sample was incubated with 50 μ l of a 100 mM sulfanilic acid solution and 50 μ l of a 10 mM *N*-(1-Naphthyl) ethylene diamine di-hydro-chloride solution. The mixture was allowed to react for 15 min, and the absorbance value at 540 nm was determined. Every sample was analyzed in triplicate. Fresh solutions of sodium nitrite were regularly prepared as standards. The reductase activity of full-length NOS2 and its carboxy-terminal domain was determined using cytochrome c as an electron acceptor after the increase in the absorbance at 550 nm at 37°C (Rodríguez-Crespo *et al.*, 1996).

Interaction of Recombinant NOS2 Containing a Carboxy-terminal -ATRL Sequence with a Cellular Lysate of A549 Cells

We performed the recombinant expression of mouse mNOS2 and purified the protein with calmodulin bound as reported above. Approximately, 1 mg of pure protein was dialyzed in order to eliminate the imidazole, and it was then allowed to bind to a Ni-NTA resin that interacts with its hexa-His N-terminal tag of NOS2. Two confluent F75 of A549 cells were scrapped and lysed in 25 mM Tris buffer, 50 mM NaCl, pH 7.5, in the presence of protease inhibitors. After centrifugation of the cell lysate at 6000 $\times$ g for 5 min at 4°C, the supernatant was loaded in the column where NOS2 was bound. The column was subsequently washed with 10 column volumes of the same buffer with protease inhibitors and then 5 volumes of 25 mM Tris buffer, 100 mM NaCl, and 10 mM imidazole, pH 7.5. The recombinant NOS2 bound to cellular proteins was eluted with 200 mM imidazole, dialyzed against 100 mM (NH₄)HCO₃, pH 8.5, and loaded in a PAGE-SDS gel for further analysis.

Construction of the GFP Fusion Proteins Plasmids and Mutagenesis

We have described the cloning and expression of the full-length wild-type NOS2, as well as NOS2-green fluorescent protein (GFP). Both of these constructs result in palmitoylated proteins at Cys3 when transfected in mammalian cells (Navarro-Lérida *et al.*, 2004b, 2006) because they have the initial sequence MACPWKFL... Site-directed mutagenesis was used in order to introduce the desired mutations, and we created the C3S and Myr constructs as described in Navarro-Lérida *et al.*, 2004b, 2006. Every mutant was obtained as a full-length NOS2-GFP chimera and as a NOS2-(1-94)-GFP chimeras. The carboxy-terminal Δ 18 NOS2 deletion mutant was constructed by standard PCR procedures and confirmed by automated sequencing.

Yeast Two-Hybrid Screens

The carboxy-terminal 10 amino acids of mouse mNOS2 (-ALEEPKGTRL-COOH) and human hNOS2 (-FPSSLEMSAL-COOH) were fused in frame to the binding domain of GAL4 in the pGBT9 vector. These stretches were assayed as preys in a yeast two-hybrid screen against PDZ domain-containing proteins that were fused in frame with the activation domain of GAL4 in the pGAD or pACT2 vectors (Clontech). The final 10 amino acids of human heart NOS2 (ALEEPKGTRL-COOH) was also fused in frame with the binding domain of GAL4 and was used to transform Y190 yeasts (MAT α). Then, these yeasts were analyzed for positive interactions against a human heart library in Y187 (MAT α). Yeasts were allowed to mate and positive colonies were selected when plated in Leu-/Trp-/His-/SD plates in the presence of 10 mM 3-amino triazole (TDO plates). Approximately 100 positive colonies were picked, and the interaction was confirmed by white/blue screening using X-Gal as a substrate. Then, the DNA of the yeasts that displayed a positive interaction was purified and amplified by PCR using oligonucleotides that annealed in the pACT2 plasmid. The PCR fragments were subsequently sequenced and analyzed using the public databases.

Laser Confocal Microscopy

Cells grown on 0.2% gelatin-coated glass coverslips and in 0.4 μ M Transwell (Costar, Acton, MA) were washed two times with phosphate-buffered saline (PBS) and fixed for 15 min at room temperature with freshly prepared 2% paraformaldehyde in PBS. The stock paraformaldehyde solution was prepared at 4% in PBS and was centrifuged at 15,000 rpm for 5 min at room temperature in a table-top microcentrifuge in order to remove insoluble material before dilution. After removal of the 2% paraformaldehyde solution, the cells were washed with PBS and incubated with cold methanol at -20°C for 10 additional minutes. The methanol was removed and the coverslips were allowed to dry for 5 min. Then, the cells were washed with PBS and incubated with the desired primary antibodies at 37°C . In general, an overnight incubation with the primary antibodies at a 1:200 dilution in a wet chamber followed by a 2-h incubation with the secondary antibody. The samples that contained phalloidin were fixed for 10 min with 2% paraformaldehyde and washed extensively with PBS followed by a 10-min incubation with 0.1% Triton X-100. After additional washes with PBS the samples were incubated with phalloidin (1:40) for 30 min at room temperature.

Finally, the slides were mounted using Fluoroguard anti-fade reagent (Bio-Rad, Richmond, CA). The subcellular localization was observed under a Bio-Rad Radiance 2100 confocal microscope, using the excitation wavelength of 405 nm for the Hoechst fluorescence (nuclei staining), 488 nm for the Cy2 fluorescence, and 543 nm for the Cy3 fluorophore. A 60 $\times$ oil immersion objective was used for the coverslip samples and a 60 $\times$ water objective was used for the Transwells. Analysis of the pictures was performed with the Confocal Assistant software (Free software by Todd Clark, version 4.02) as well as with Laserpix and Lasersnap software from Bio-Rad. Overlap of green and red labeling is depicted yellow; overlap of green and blue labeling is depicted light blue; overlap of red and blue is depicted violet.

Tissue Immunostaining

Murine livers were extracted and frozen at -20°C , and serial 10- μm -thick sections were cut with a Leitz sledge microtome (Wetzlar, Germany) onto gelatinized glass coverslips. The preparations were fixed in a 3.7% paraformaldehyde solution in PBS, pH 7.4, for 45 min at room temperature, washed with PBS, and permeabilized with cold acetone for 15 min at room temperature. The sections were incubated overnight with the indicated antibodies in PBS at 4°C . The sections were incubated with fluorescent secondary antibodies (labeled with Cy2 or Cy3) and treated with Hoechst 33258 for 30 min at room temperature. Fluorescence was visualized on a MRC 1024 microscope (Bio-Rad) with Lasersnap software.

Gene Silencing Using Interference RNA

All the gene silencing experiments were performed on MDCK cells, either polarized or not, using various concentrations (100–500 nM) of the synthetic interference RNA (iRNA): CAP70 sense rGrArGrCrArGrGUUUrGrArGUrGrAUrATT in a duplex with CAP70 antisense UrAUrCrArCrUrCrArArArCrCUUrGrCUrCIT purchased from Prologo (Kyoto, Japan). The iRNA was transfected using Escort IV (Sigma), and the amount of CAP70 was determined by Western blot. A scrambled iRNA was also added as a control. The DNA corresponding to wild-type NOS2 or the carboxy-terminus Δ 18 deletion mutant was added at the same time than the iRNA. Nitrites in the medium and protein levels of CAP70, β -tubulin, and NOS2 were determined 36 h after transfection.

RESULTS

NOS2-mediated $\cdot\text{NO}$ Release Occurs in the Apical Domain of Polarized Madin-Darby Canine Kidney Cells

NOS2 is known to become associated with the apical side of primary human proximal tubular epithelial cell cultures treated with cytokines, and the concentration of the enzyme in this region results in the selective release of $\cdot\text{NO}$ to the apical medium (Glynn *et al.*, 2002). Hence, we decided to compare the subcellular distribution as well as the release of $\cdot\text{NO}$ of both wild-type NOS2 as well as NOS2 with the GFP reporter fused to its carboxy-terminal end. With that in mind, we plated Madin-Darby canine kidney (MDCK) cells in Transwell chambers, transfected them with the desired construct, and allowed the cells to reach confluence. As shown in Figure 1A, wild-type NOS2 transfected in MDCK cells excluded from the cell nucleus and gave a cytosolic phenotype with a strong tendency to localize toward the apical face. This specific subcellular localization resulted in the enrichment of the released $\cdot\text{NO}$ into the apical side (84% against 16% in the basolateral side). Conversely, if we blocked the carboxy-terminal end of NOS2 fusing the GFP

Figure 1. Transfected mNOS2 and mNOS2-GFP display different subcellular distribution in polarized MDCK cells and release $\cdot\text{NO}$ differently in the apical and basolateral compartments. MDCK cells were grown in Transwells and transfected with mNOS2 (A) or mNOS2-GFP (B). The subcellular distribution of each construct was analyzed using laser confocal microscopy. The mNOS2 fluorescence was obtained using a Cy2-labeled secondary antibody and is shown in green. The basolateral staining is shown using antibodies against β -catenin and a Cy3-labeled secondary antibody. In B the GFP reporter is fused to the carboxy-terminal end of NOS2, hence blocking the binding of its four last residues to proteins containing PDZ domains. Both the upper and bottom chambers contained 500 μl of medium. Thirty hours after transfection the release of $\cdot\text{NO}$ to the medium was determined in both chambers using the Griess method. The total amount of $\cdot\text{NO}$ released in both chambers was considered 100%, and the relative distribution is plotted in the right panels. Bas, basolateral (bottom chamber); Ap, apical (upper chamber). The XZ and YZ confocal planes are shown at the bottom and right of the XY plane, respectively. A single transfected cell is enlarged in all cases. Bar, 50 μm . The data shown are representative of three independent experiments; mean $\pm$ SD.

reporter in frame, the subcellular distribution was clearly altered (Figure 1B). Although this construct also excluded from the nucleus and gave a cytosolic pattern, it did not concentrate toward the apical face. Interestingly, a certain colocalization with the basolateral marker β -catenin could also be observed. This GFP-tagged NOS2 released 67% of the total $\cdot\text{NO}$ toward the basolateral chamber and 33% toward the apical chamber (Figure 1B). Hence, when NOS2 is expressed in polarized cells, it adopts a mostly apical phenotype that results in the vectorial release of $\cdot\text{NO}$ to the apical chamber.

EBP50 Binds to NOS2 Isoforms That Contain Either the -ATRL-COOH or -MSAL-COOH Carboxy-terminal Tetrapeptides

With the intention of identifying novel proteins with PDZ domains that might interact with the last four residues of human heart NOS2 (GTRL-COOH) or with murine NOS2 (GTRL-COOH in rat and ATRL-COOH in mouse) we cloned

Figure 2. EBP50 binds to mNOS2 and hNOS2 and in both cases increases NOS2 activity. (A) The final 10 amino acids of both human heart NOS2 (with GTRL being the final four residues) and the final 10 amino acids of human hepatic NOS2 (with MSAL being the final four residues) were fused in frame with the binding domain of GAL4 and were screened against full-length or shorter constructs of EBP50 fused to the activation domain of GAL4 in a yeast two-hybrid screen. The intensity of the interaction: +++, very strong; ++, strong; or –, absence of interaction. (B) EBP50 was fused in frame with GST and expressed in recombinant form in *E. coli*. The purified protein was analyzed by SDS-PAGE and Coomassie blue staining (left). This recombinant GST-EBP50 was allowed to interact with a cytosolic extract of macrophages activated with cytokines. The binding of EBP50 to mNOS2 was determined by Western blot using anti-NOS2 antibodies (B, right). (C) Purified recombinant hNOS2 and mNOS2 were incubated with a fivefold molar excess of GST-tagged recombinant EBP50 and both the $\cdot$ NO synthesis activity and the cytochrome c reduction activity were determined at 37°C (□). The activity was referred to 100% in the absence of added EBP50 (■); mean $\pm$ SD; * p < 0.05 versus absence of GST-EBP50.

the final 10 residues of human heart NOS2 in the bait plasmid pGBT9. This plasmid was used to transform yeasts that were later allowed to mate with a human heart library. Three independent positive clones were isolated that contained the sequence of human EBP50 as an interacting partner. This protein possesses two PDZ domains and has been previously shown to interact with the carboxy terminus sequence MSAL-COOH present in human hNOS2 (Glynn *et al.*, 2002). Consequently, our data indicate that EBP50 interacts not only with human hNOS2 but also with human heart NOS2. Inspection of the affinity of EBP50 toward the carboxy-terminal end of both human NOS2 isoforms was performed in the galactosidase assay of the yeast two-hybrid screen. Thus, in addition to the human heart NOS2 (ALEEP-KGTRL-COOH), we also cloned the 10 final amino acids of human hNOS2 (QPSSLEMSAL-COOH) in frame with the binding domain of GAL4 and analyzed their binding to full-length EBP50 or to PDZ2 of EBP50 (Figure 2A). A very strong interaction was observed in the case of the heart isoform and a strong interaction in the case of its hepatic counterpart. Deletion of PDZ1 domain was sufficient to abrogate the binding of EBP50 to the carboxy-terminal end of both proteins. Hence, EBP50 can interact with the carboxy-terminal end of proteins containing both TRL-COOH and SAL-COOH, which are included within type I motifs of proteins that bind to PDZ domains.

To confirm the interaction with proteins that contained TRL-COOH as carboxy-terminal amino acids, we expressed and purified recombinant EBP50 fused to the GST fusion protein to homogeneity (Figure 2B). This recombinant protein was bound to a reduced glutathione (GSH)-agarose resin and a lysate of Raw 264.7 macrophages challenged with a mixture of LPS/IFN- γ was flowed through. The column was subsequently washed extensively and the GST-tagged EBP50 was eluted with GSH. As shown in Figure 2B, our Western blot results using anti-NOS2 antibodies revealed that mouse mNOS2, with the TRL-COOH carboxy-terminal sequence bound, albeit not tightly, to EBP50, as shown by the significant amount of NOS2 that flowed through the resin without binding to EBP50 (data not shown). Our data indicate that EBP50 probably adopts a proper folding inside mammalian cells and yeasts and interacts with NOS2 efficiently. However, when EBP50 is expressed in bacteria, the binding to NOS2 is remarkably weak, in accordance with previous data reported for hNOS2

(Glynn *et al.*, 2002). No binding to the resin was observed when GST alone was tested for interaction with mNOS2 (data not shown).

Because the carboxy-terminal stretch of NOS2 is known to modulate the electron transfer from the reductase domain toward the heme-oxygenase of the protein (Roman *et al.*, 2000), we decided to inspect if EBP50 binding resulted somehow in changes in the $\cdot$ NO synthesis by NOS2. Recombinant expression of human hepatic and mouse mNOS2 was performed in *E. coli* in a two-plasmid coexpression system with calmodulin that we had previously optimized (Rodríguez-Crespo and Ortiz de Montellano, 1996). Addition of a fivefold molar excess of GST-EBP50 over NOS2 increased $\cdot$ NO synthesis of both human hNOS2 and mouse mNOS2 by 41 and 56%, respectively (Figure 2C), whereas GST alone resulted in no changes in activity (data not shown). When we inspected the changes obtained in cytochrome c reduction for both proteins in the presence of GST-EBP50, we could detect an increase in 12% for human hNOS2 and 30% for mouse mNOS2. Because the cytochrome c reduction assay reflects the total activity of the carboxy-terminal reductase domain of nitric oxide synthases, the increase in $\cdot$ NO synthesis in the presence of EBP50 must reflect, at least partially, an increased electron transfer from the reductase toward the heme-oxygenase domain.

Identification of CAP70 as a Novel NOS2-interacting Protein

We next decided to identify novel proteins that might bind to NOS2 isoforms with the carboxy-terminal triplet TRL-COOH. Therefore, we expressed recombinant NOS2 with TRL-COOH as the carboxy terminal residues and passed it through a Ni-NTA resin that binds to the hexa-His-tagged positioned at the N-terminal end. Subsequently, human lung, polarized A549 cells were grown in culture and a lysate of these cells was loaded in the column. After extensive wash, NOS2 in complex with interacting proteins was eluted with imidazole and loaded in a PAGE-SDS gel that was stained with Coomassie blue. The most prominent bands corresponding to NOS2-interacting partners appeared at ~67 and ~70 kDa as well as 55 kDa (Figure 3A, bands marked with asterisks). Interestingly, we suspected that the 70-kDa band corresponded to CAP70, a protein with four PDZ domains known to bind to the cystic fibrosis conductance regulator that concentrates partially in apical

Figure 3. Identification of CAP70 as a mNOS2-associated protein expressed in multiple polarized cell lines. (A) Macrophage NOS2 (mNOS2) that was recombinantly expressed and purified using two affinity resins appeared in a Coomassie blue-stained gel as a clear 135-kDa band (A, second lane). At the same time, during the purification of recombinant mNOS2 a cellular lysate of A549 cells was incubated with the protein bound to the 2', 5'-ADP-agarose resin. Finally, mNOS2 in a complex with cellular-interacting proteins was eluted and analyzed by Coomassie blue staining (A, third lane). Bands that appeared as mNOS2-interacting proteins are marked with asterisks. Because a prominent band appeared at ~70 kDa, this sample was analyzed by Western blot and immunodetected using anti-CAP70 antibodies (A, lane marked I.D.). (B) Recombinant GST-CAP70 was purified using a GSH-agarose

resin, and its purity was confirmed by Coomassie blue staining of the gel. (C) Approximately 200 μ g of recombinant hexa-His tagged mNOS2 were incubated with 200 μ g of recombinant GST-tagged CAP70 for 30 min at room temperature. Half of the mixture was then loaded in a Ni-NTA-Agarose column and the other half in a GSH-Agarose column. The columns were extensively washed and bound proteins were eluted with either GSH (for the GSH-Agarose resin, top) or with imidazole (for the Ni-NTA-Agarose resin, bottom). The recombinant mNOS2 and GST-CAP70 are marked with arrows. (D) Expression of endogenous CAP70 in A549, CaCo2, and MDCK cells, all of which polarize when grown confluent in transwells. A positive control of recombinant CAP70 that migrates at ~70 kDa in SDS-PAGE is shown in the lane marked with an arrow. (E) Colocalization of CAP70 and mNOS2 in A549 cells analyzed by laser confocal microscopy. Cell nuclei were stained with Hoechst, transfected mNOS2 was immunolocalized using a Cy2-labeled secondary antibody, and endogenous CAP70 was stained using a Cy3-labeled secondary antibody. Bar, 50 μ m.

membranes (Wang *et al.*, 2000). In fact, when we analyzed the proteins that interacted with NOS2 by Western blot, we were able to immunodetect CAP70 (Figure 3A). To confirm the interaction between NOS2 and CAP70, we expressed GST-tagged CAP70 and purified this recombinant protein to homogeneity (Figure 3B). Recombinant hexa-His-tagged mNOS2 was incubated with recombinant GST-tagged CAP70 for 30 min at room temperature. Half of the mixture was then loaded in a Ni-NTA-agarose column and the other half in a GSH-agarose column. The columns were washed extensively, and the bound proteins were eluted with GSH in the case of the GSH-agarose or imidazole in the case of the Ni-NTA resin. Fractions corresponding to the first three tubes that were eluted were run in a SDS-PAGE and are shown in Figure 3C. On the other hand, GST alone was unable to interact with NOS2 (data not shown). Thus, in accord with the observed interaction of CAP70 present in the cellular lysate, recombinant CAP70 also interacted robustly with NOS2. Expression of CAP70 in polarized cells in culture was confirmed for A549, CaCo2, and MDCK cells, even though CaCo2 seemed to express an isoform of the protein with slightly smaller molecular mass (Figure 3D). Purified recombinant CAP70 was analyzed as a control of correct migration. When we inspected the distribution of transfected mNOS2 in A549 cells, we could conclude that CAP70 and mNOS2 colocalized throughout the cytosol, although mNOS2 also stained perinuclear areas of the cell where CAP70 was absent (Figure 3E).

CAP70 Binds to the Three Carboxy-terminal Amino Acids of NOS2 and the Binding Results in Increased Activity

Then, we analyzed the binding of CAP70 to NOS2 using the yeast two-hybrid technique. We utilized the constructs where the final 10 carboxy-terminal amino acids of either human heart NOS or human hepatic NOS were fused in frame with the GAL4 binding domain crossing them against wild-type CAP70 or a deletional mutant (Figure 4A). The positive interaction observed for both the carboxy-terminal stretch of human heart and hNOS2 confirmed that CAP70

did in fact associate with this region of NOS2. Deletion of PDZ2, PDZ3, and PDZ4 abrogated completely the binding, hence revealing that PDZ1 by itself is unable to provide a fully positive interaction.

When we used the recombinant GST-CAP70 described above and analyzed changes in the $\cdot$ NO releasing activity, we were able to observe a 16% increment in the case of hNOS2 and a 52% increment for mNOS2 (Figure 4B). No changes in NOS2 activity were observed when GST alone was added to the reaction mixtures (data not shown). This changes were in agreement with the differential binding strengths observed in the yeast two-hybrid screen reported above, in which NOS2 with the TRL-COOH sequence interacted more efficiently with CAP70 than NOS2 with the SAL-COOH sequence. On the other hand, the changes observed in the NOS2 cytochrome c reduction rates were more discrete, 7% for hNOS2 and 15% for mNOS2 (Figure 4B). Because the total increase in $\cdot$ NO synthesis could be rationalized only partially with the observed increase in cytochrome c reduction activity, we wondered if CAP70 might be promoting the transition of inactive monomeric mNOS2 into its active dimers. We decided to inspect the monomer-dimer equilibrium of mNOS2 using pseudonative SDS-PAGE. Although this technique is more effective when analyzing the dimerization of both NOS1 (Klatt *et al.*, 1995) or NOS3 (Rodríguez-Crespo *et al.*, 1996), we have recently used this technique when analyzing the monomer to dimer ratio of NOS2 (Navarro-Lérida *et al.*, 2006). Incubation of mNOS2 (M_r 135,000 Da) with increasing amounts of recombinant CAP70 resulted in an elevation in the population of dimers (M_r ~270,000 Da) that could be observed in the gel (Figure 4C). This monomer-to-dimer shift was more apparent after densitometric scanning of the gels. GST alone was unable to induce the dimerization of mNOS2 (data not shown). Consequently, CAP70 binding is either promoting the dimerization of NOS2 or stabilizing the population of dimers, a process known to result in increased $\cdot$ NO synthesis.

Finally, after demonstrating the interaction of CAP70 with both mNOS2 and hNOS2 using recombinant proteins and in

Figure 4. (A) CAP70 associates with both mNOS2 and hNOS2 and increase their $\cdot\text{NO}$ synthesis. The final 10 amino acids of either mNOS2 (being the last four ATRL) or hNOS2 (being the last four MSAL) were fused in frame with the binding domain of GAL4 and screened in a yeast two-hybrid assay for interaction with two constructs of CAP70 fused in frame with the GAL4 activation domain. The degree of interaction is shown to the right: ++, a strong positive interaction; +, a positive interaction; and +/-, a weak interaction. (B) Purified recombinant hNOS2 and mNOS2 were incubated with a fivefold molar excess of GST-tagged recombinant CAP70, and both the $\cdot\text{NO}$ synthesis activity and the cytochrome c reduction activity were determined at 37°C (■). The activity was referred to 100% in the absence of added CAP70 (□). The activity was $p < 0.05$ versus absence of CAP70. (C) Purified recombinant mNOS2 was run in a pseudonative SDS gel on ice in order to maintain the dimer partially intact. Then a 2.5, 5, 10, and 20 M excess of recombinant CAP70 was added, and the increase in the population of dimeric mNOS2 was determined staining the gel with Coomassie blue. The positions where the CAP70-GST, the monomeric mNOS2 and the dimeric mNOS2 appear in the gel are shown by arrows. A densitometric analysis of each lane is also shown at the bottom. Identical results were obtained in three independent experiments. (D) mNOS2 was transfected in A549 cells and 48 h after transfection, mNOS2 was immunoprecipitated using anti-NOS2 antibodies. The immunoprecipitated complexes were immunodepleted using anti-NOS2 antibodies (top) and against anti-CAP70 antibodies (bottom).

the yeast two-hybrid assay, we inspected if this association also took place inside the cell. Accordingly, A549 cells were transfected with mNOS2 and 48 h after transfection the cells were immunoprecipitated with anti-NOS2 antibodies. Subsequently, the presence of both mNOS2 and CAP70 was confirmed in the immunoprecipitated mixture (Figure 4D). This piece of data corroborates the interaction between NOS2 and CAP70.

CAP70 Is a Cytosolic Protein That Excludes from the Basolateral Membranes and Becomes Enriched in the Apical Surface

Laser confocal immunofluorescence analysis of the staining pattern in polarized A549, CaCo2, and MDCK cells revealed that the distribution of β -catenin, a basolateral marker, was completely opposite to that showed by CAP70 (Figure 5A). Although β -catenin distributed in the basolateral membranes and in cell-cell contacts in polarized A549, CaCo2, and MDCK cells, CAP70 specifically avoided this localization. However, CAP70 did not stain exclusively the apical surface but instead it also distributed throughout the cytosol. This immunodistribution is in agreement with the staining displayed by CAP70 in kidney and intestine tissues (Wang *et al.*, 2000). When the orthogonal projection of MDCK cells transfected with a NOS2 construct was analyzed, we could observe an almost complete overlap (yellow) in the NOS2 (green) and CAP70 (red) signals (Figure 5B). Because CAP70 avoids the basolateral localization, the cells displayed a “teeth-like” immunostaining. NOS2 excluded specifically from the nucleus and from the basolateral membrane, stained the cytosol, and concentrated toward the apical surface. Treatment of NOS2-transfected MDCK cells grown in Transwells for 1 h with 10 μM cytochalasin-D, an inhibitor of actin polymerization, decreased the ratio of $\cdot\text{NO}$ released to the apical chamber from 84 to 52%, whereas the $\cdot\text{NO}$ released to the basolateral chamber increased from 16 to 48%. This result clearly indicates that the integrity of the actin microtubules is necessary for the correct apical delivery of $\cdot\text{NO}$ in polarized tissues.

Down-Regulation of the CAP70 Protein Levels using iRNA

We next decided to use iRNA and reduce the protein levels of CAP70 in cultured mammalian cells with the intention of analyzing the effect of this decrease on the release of $\cdot\text{NO}$ both in terms of total amount as well as targeting to apical/basolateral membranes. Consequently, we transfected MDCK cells with NOS2 in the presence of increasing

Figure 5. CAP70 excludes from the basolateral membrane and colocalizes with NOS2 in polarized cells. (A) A549, Caco2, and MDCK cells were grown in Transwells and after polarization were stained with antibodies against the basolateral marker β -catenin (top) or against CAP70 (bottom). The staining of both β -catenin and CAP70 was obtained using a rabbit primary antibody followed by incubation with a Cy3-labeled secondary antibody. (B) A549 polarized cells were transfected with NOS2, and the colocalization with CAP70 was determined. The staining of NOS2 was obtained using a mouse anti-NOS2 antibody followed by incubation with a Cy2-labeled secondary antibody, whereas the labeling of CAP70 was obtained using a rabbit anti-CAP70 antibody followed by incubation with a Cy3-labeled secondary antibody. The XZ and YZ confocal planes are shown at the bottom and right of the XY plane, respectively. Cell nuclei are stained with Hoechst. Bar, 50 μm . The data shown are representative of three independent experiments.

Figure 6. Gene silencing of CAP70 using a specific iRNA. (A) Increasing amounts of iRNA and fixed amounts of a NOS2 plasmid were used to transfect MDCK and the protein levels of CAP70, β -tubulin, and NOS2 were determined by Western blot 36 h after transfection (left). At the same time the supernatants were collected and the amount of $\cdot$ NO released was determined using the Griess assay. A scrambled iRNA was also added as a control. (B) In a separate experiments, MDCK were seeded in Transwells and NOS2 was transfected in the presence or absence of the iRNA. The amount of $\cdot$ NO released was determined 36 h after transfection using the Griess assay. A constant volume of 500 μ l of medium was maintained in both chambers. A carboxy-terminal Δ 18 deletion mutant that lacked the GTRL or MSAL carboxy-terminal motifs was constructed, and it was used to transfect polarized MDCK in the presence or absence of the CAP70 iRNA (right). Likewise, the amount of the released $\cdot$ NO was determined in each chamber using the Griess assay.

amounts of a CAP70-directed iRNA. The addition of 500 nM iRNA resulted in $\sim$ 30% of the amount of CAP70 in the absence of the iRNA (Figure 6A, left). No significant changes were observed in the protein levels of β -tubulin or NOS2. When we measured the amount of released $\cdot$ NO in the supernatant, we observed that the iRNA decreased the total amount of $\cdot$ NO to 48% of the levels obtained in the absence of added reagent (Figure 6A, right). When we used an iRNA with a scrambled sequenced, we were able to observe a modest decrease in the total $\cdot$ NO detected (88% of the levels in the absence of added reagent). Hence, binding of CAP70 to NOS2 within MDCK cells increases the total $\cdot$ NO-synthesizing activity of the latter.

To inspect if the decrease in the CAP70 protein levels affected the selective release of $\cdot$ NO toward the apical or basolateral membranes, MDCK cells were grown in Transwells and were transfected with NOS2 in the presence or absence of iRNA (Figure 6B, right). When we determined the amount of $\cdot$ NO in each chamber 36 h after transfection we observed that 500 nM iRNA changed the basolateral-apical ratio from 44 to 76% in the absence to 59 to 41% in the presence of this reagent. This result clearly indicates that

CAP70 determines the correct apical delivery of NOS2 in polarized cells.

Because NOS2 can accept moderate amino acid deletions in the carboxy-terminal end and still maintain enzymatic activity (Roman *et al.*, 2000), we also deleted the final 18 amino acids of NOS2 with the intention of removing the -TRL motif that might interact with proteins with PDZ domains such as EBP50 or CAP70. When this deletion mutant of NOS2 was transfected in polarized MDCK cells grown in Transwells, it delivered 44% of the $\cdot$ NO toward the basolateral chamber and 56% toward the apical chamber (Figure 6B, right). No significant changes were observed in this mutant in the presence of iRNA for CAP70. Thus, these carboxy-terminal amino acids clearly control the proper subcellular targeting of NOS2.

Inspection of the N-terminal Signals on NOS2 Targeting

We have previously shown that NOS2 is palmitoylated on Cys3, in a process completely necessary for the progression along the sorting pathways (Navarro-Lérída *et al.*, 2004b, 2006). When we expressed a Cys3Ala mutant or used Br-palmitate in order to abrogate palmitoylation of NOS2, the protein accumulated in perinuclear areas and its activity was diminished considerably. Nonetheless, we have investigated if palmitoylation of NOS2 at Cys3 is also involved with apical targeting in polarized MDCK cells. With that in mind, we analyzed the subcellular localization of several NOS2 constructs that had the GFP reporter fused to their C-terminal end, hence abrogating their interaction with proteins involved in apical targeting such as EBP50 or CAP70. Transfection of wild-type or C3S NOS2(1–94)-GFP in MDCK resulted in a diffuse cytosolic and nuclear staining, but the protein did not accumulate toward the apical membrane (Figure 7). Although the wild-type construct displayed certain basolateral staining and a patent colocalization with the β -catenin marker (overlap is depicted yellow), the C3S mutant specifically excluded from the basolateral membrane. Introduction of a surrogate myristoylation site at the N-terminus in both short (1–94) or full-length-tagged constructs resulted in a cytosolic pattern together with a significant colocalization with the basolateral marker β -catenin (overlap is depicted yellow) but no apical targeting (Figure 7). Quantification of the green-red overlap was performed using the orthogonal planes in each of the four constructs that were transfected. This analysis allowed us to determine that $21 \pm 5\%$ of the wt-NOS2(1–94)-GFP fluorescence overlapped with the red fluorescence of β -catenin. The numbers that we obtained were 1% overlap for the C3S-NOS2-GFP, $26.5 \pm 6\%$ overlap for the Myr(1–94)-GFP and $23 \pm 8\%$ overlap for the Myr-NOS2-GFP construct. In fact, engineering a surrogate myristoylation site at the N-terminal end of NOS2 with the GFP reporter fused to the carboxy-terminal end of the protein restored NOS2 activity and resulted in a protein in which 72% of $\cdot$ NO was released toward the basolateral chamber and 28% toward the apical chamber. These results establish that apical targeting is mediated solely by the interaction of the final four amino acids of NOS2 through their association with EBP50 or CAP70 and eventually with cortical actin filaments. On the other hand, the N-terminal acylation of NOS2 is involved in intracellular traffic and exit from the Golgi-trans-Golgi network, a process needed for full activity of NOS2. To analyze the effect of palmitoylation on the apical sorting of NOS2 in polarized cells, wild-type full-length NOS2 was transfected in MDCK and the palmitoylation inhibitor 8-Br-palmitate was added. This treatment reduced the total amount of $\cdot$ NO released into the medium to $\sim$ 15% of the total amount observed in the absence of

Figure 7. Immunolocalization of GFP-tagged NOS2 mutants transfected in polarized MDCK cells. Three NOS2(1–94)-GFP constructs (wild-type protein, C3S, and Myr mutants) together with a full-length Myr-NOS2-GFP mutant were transfected in polarized MDCK cells, and the subcellular distribution was analyzed by laser confocal microscopy. The basolateral membrane of the cells was stained with an anti- β -catenin primary antibody followed by a Cy3-labeled secondary antibody. The GFP fluorescence was obtained after excitation at 488 nm. The XZ and YZ confocal planes are shown at the bottom and right of the XY plane, respectively. A single transfected cell is enlarged in all cases. Bar, 50 μ m. The data shown are representative of three independent experiments.

treatment. In addition, this inhibition of NOS2 palmitoylation resulted in the loss of the selective release of $\cdot$ NO toward the apical chamber. Hence, as in the case of COS7 cells transfected with NOS2 (Navarro-Lrida *et al.*, 2004b, 2006), palmitoylation is a prerequisite for $\cdot$ NO synthesis and correct transit through the sorting pathways.

In Vivo Polarization of NOS2 in Liver Tissues

We next decided to inspect if EBP50 and NOS2 displayed a polarized distribution in murine liver tissues. Control experiments were first performed using untreated animals and antibodies against EBP50 were used. As shown in Figure 8A, cortical actin staining labeled beautifully the canaliculi, hence revealing the apical hepatocyte membrane. EBP50 antibodies gave a nonspecific nuclear staining, but they also localized to apical membranes (arrows in Figure 8A). To analyze the NOS2 distribution in the liver, mice were injected intraperitoneally with bacterial lipopolysaccharide (LPS) as previously described (Navarro-Lrida *et al.*, 2004a) in order to induce an inflammatory condition. Polarization of hepatocytes involves formation of functionally distinct sinusoidal (basolateral) and canalicular (apical) plasma membrane domains that are separated by tight junctions. Because the canalicular membrane is highly enriched in actin filaments, F-actin staining with fluorescent phalloidin can be used to visualize this membrane subdomain (Zegers *et al.*, 1998; de Marco *et al.*, 2002). Inspection of the immunodistribution of NOS2 in liver tissue was performed in

10- μ m sections stained with a Cy3-labeled secondary antibody (red) together with Hoechst (nuclei staining, blue) and Alexa 488-phalloidin (F-actin, green; Figure 8). In hepatocytes, actin staining mostly distributed in the bile canaliculus (marked C in Figure 8), whereas it excluded from the sinusoidal membranes (marked S in Figure 8). Analysis in detail of NOS2 distribution within hepatocytes at a higher magnification revealed that actin staining was more evident toward the apical side of the polyhedral hepatocytes (Figure 8, green staining, arrows). Likewise, NOS2 staining, although positive throughout the cytoplasm, was significantly more intense in the canalicular membranes. Remarkably, Kupffer cells/macrophages seemed to express large amounts of NOS2, according to the significant staining that could be observed (marked with asterisks).

DISCUSSION

NOS2 is usually transcriptionally up-regulated in response to various proinflammatory stimuli. Although macrophages are usually the cellular model for NOS2 studies, many polarized cells also respond to cytokines, hence expressing large amounts of NOS2 in their apical surface that results in the release of $\cdot$ NO to the extracellular medium (Rumbo *et al.*, 2005). In this article we have addressed the mechanisms by which NOS2 becomes associated with proteins that have PDZ domains and are transported toward the apical side of the cell.

Interestingly, two isoforms with distinct carboxy-terminal sequences can be found in human tissues. The hepatic isoform has been previously shown to associate to EBP50 and become transported toward the apical side of cytokine-treated epithelial cells (Glynn *et al.*, 2002). With that in mind, we fused the final 10 residues of the human heart isoform to the binding domain of GAL4 and used it as a bait in a yeast two-hybrid screen using a human heart library. Through this methodology we were able to retrieve EBP50 as a NOS2-interacting protein as well. Therefore, EBP50 is capable of interacting with both isoforms of human NOS2, as long as the type I motif for interacting with PDZ domain-containing proteins is present in the carboxy-terminal end of them.

To identify novel proteins that associate with the carboxy-terminus sequence TRL-COOH, we expressed recombinant mNOS2 and after binding it to a Ni-NTA affinity resin proteins present in a cellular lysate of polarized A549 were allowed to bind. Using this approach, we were able to identify CAP70 as a novel NOS2-interacting protein. According to our data, CAP70 also binds to the carboxy-terminal end of proteins that contain a type I consensus binding site. Remarkably, we show herein that CAP70 augments significantly the $\cdot$ NO synthesizing activity of both hNOS2 and mNOS2 and also promotes the dimerization of the protein, a process known to be indispensable for activity.

In our hands, wild-type NOS2 displayed a cytosolic distribution with a strong staining of the apical membrane of polarized A549 and MDCK cells. Although we were unable to detect apical staining exclusively, our results indicate that when the carboxy-terminal stretch of NOS2 is free to interact with either EBP50 or CAP70 these proteins withdraw NOS2 from the proximities of the basolateral membrane and permit the selective release of $\cdot$ NO to the apical chamber.

Polymerized actin filaments have been reported to appear at the membrane surface of phagosomes (Defacque *et al.*, 2000; Miller *et al.*, 2004). In macrophages, the binding of NOS2 to PDZ domain-containing proteins that couple to the actin filaments seems to be indispensable for phagocytosis to

Figure 8. Polarized distribution of NOS2 and EBP50 in hepatocytes. (A) A fresh murine liver was frozen 10- μ m sections were obtained in a cryotome as described in *Materials and Methods*. The slices were processed for immunostaining using Hoechst (nuclei staining, blue), Alexa488-phalloidin (labeling of the canalicular tubules, green), and antibodies against EBP50 (red). The apical immunostaining of EBP50 is depicted with white arrows. (B) To induce NOS2 expression in the liver, mice were intraperitoneally injected with LPS, and 48 h after injection the immunostaining of induced NOS2 was determined in liver sections. Bile canaliculi are marked C, the sinusoids are marked S, and both of them can appear transversely or longitudinally depending on the shape of the tissue at any specific point. Actin present in the apical (canalicular) membrane of cells was stained with Alexa488-phalloidin and is shown in green. NOS2 immunofluorescence was determined using a primary antibody followed by a Cy3-labeled secondary antibody and is shown in red. Nuclei were stained with Hoechst and are shown in blue. An area marked in the white box is shown at a higher magnification for detail. The apical membrane distribution of both actin and NOS2 is marked with white arrows. Kupffer cells/macrophages are marked with asterisks. Bar, 50 μ m.

occur. In addition, because this binding is concomitant with the increase in mNOS2 activity, large amounts of $\cdot$ NO would be released in the proximity of the phagosomes. This observation would concur with the known antiviral and antibacterial activity of NOS2. Hence, in macrophages, NOS2 is not transported toward the plasma membrane but rather toward these phagosomes that are enriched in endocytosed bacteria, in agreement with previous data (Webb *et al.*, 2001; Miller *et al.*, 2004).

The inhibition of actin polymerization using the drug cytochalasin-D resulted in a dual effect. On one hand it altered the intracellular distribution of NOS2. On the other hand, its addition resulted in a significant reduction of the total amount of $\cdot$ NO released. This was observed when NOS2 was transfected in polarized MDCK cells. Consequently, proteins that bind to the carboxy-terminal end of NOS2 mediate both the correct release at the proper site as well as the total amount of $\cdot$ NO to be released.

Finally, our data also point out that there is a parallelism between NOS2 and NOS3 when analyzing the role of the carboxy-terminal extensions. Sequence comparison between cytochrome P450 reductase and all three NOSs reveal that in NOS1, NOS2, and NOS3 there are carboxy-terminal extensions of ~33, ~22, and ~43 residues, respectively. The process involving phosphorylation of bovine NOS3 by numerous kinases on Ser-1179 (Ser-1177 in human NOS3) significantly increase the activity of the protein and has been characterized most extensively. In fact, multiple kinases that phosphorylate Ser-1179 have been identified, including AMP kinase, Akt (protein kinase B), and protein kinase A (Michell *et al.*, 1999; Bauer *et al.*, 2003 and references therein).

Mutation of Ser-1179 to aspartic acid, which mimics the phosphorylated state, results in enhanced NOS3 reductase activity and $\cdot$ NO production (McCabe *et al.*, 2000). Thus, in NOS3 these carboxy-terminal extensions function as “lids” that diminish the reductase activity of the protein and can be “opened” by phosphorylation or deletion. As in the case of NOS3, deletion of this carboxy-terminal extension of NOS2 is known to increase the reductase activity of the protein as well as the electron transfer to the heme-oxygenase domain (Xie *et al.*, 1994; Roman *et al.*, 2000). Although no phosphorylation sites have been revealed in the carboxy-terminal extension of NOS2 yet, our data indicate that an additional manner to “open the lid” and increase the electron transfer is through the binding of this extension to proteins that possess PDZ domains. Moreover, this interaction would be a mode of regulating the correct release of $\cdot$ NO at the appropriate site within the cell.

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