

## Oligomerization of Endothelial Nitric Oxide Synthase

EVIDENCE FOR A DOMINANT NEGATIVE EFFECT OF TRUNCATION MUTANTS\*

(Received for publication, July 20, 1995, and in revised form, August 28, 1995)

Christine M. Lee‡, Lisa J. Robinson§, and Thomas Michel¶

From the Cardiovascular Division, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts 02115

**Nitric oxide produced by the endothelial isoform of nitric oxide synthase (ecNOS) is a key determinant of vascular tone. In contrast to other nitric oxide synthase (NOS) isoforms, which have been characterized as soluble homodimeric enzymes, ecNOS is predominantly membrane-associated, a feature that has hindered direct biochemical analyses of its oligomeric structure. We investigated ecNOS oligomerization using co-immunoprecipitation experiments in transiently transfected COS-7 cells. When COS-7 cells co-transfected with constructs encoding wild-type ecNOS and an epitope-tagged myristoylation-deficient mutant were biosynthetically labeled with [<sup>3</sup>H]myristate, the antibody to the epitope tag specifically immunoprecipitated <sup>3</sup>H-labeled ecNOS, reflecting enzyme oligomerization. In COS-7 cells transfected with cDNAs encoding epitope-tagged truncation mutants and untagged full-length ecNOS, the wild-type enzyme could be immunoprecipitated by the antibody to the epitope tag. Co-immunoprecipitation of ecNOS with truncation mutants documented that both N- and C-terminal domains are involved in ecNOS oligomerization. When these truncation mutants are co-expressed with wild-type ecNOS, they exert a marked dominant negative effect on enzyme activity. Since NOS oligomerization itself may be subject to dynamic modulation, the regulation of ecNOS assembly may have implications for NO signaling in the vascular wall.**

Nitric oxide, a ubiquitous messenger molecule, is synthesized by a family of nitric oxide synthases (NOS)<sup>1</sup> and partici-

pates in diverse cellular processes, including neurotransmission, immune regulation, and vascular homeostasis (1). Three distinct NOS isoforms have been identified: neuronal (nNOS), inducible (iNOS), and endothelial NOS (ecNOS). These NOS isoforms subserve disparate physiological roles and differ markedly in their tissue distribution and mechanisms of regulation (see Refs. 2–4 for review). Nevertheless, the NOS isoforms appear to share many biochemical features, including common cofactor and substrate requirements. All known NOS isoforms catalyze the calmodulin-dependent oxidation of L-arginine to form nitric oxide plus L-citrulline. Recently, it was shown that dimerization of iNOS is required for catalytic activity (5); the active nNOS is also a homodimer (6). The oligomeric structure of ecNOS is unknown.

Oligomerization may serve as an important mechanism both for enzyme regulation and for signal transduction (7, 8). Dimerization of iNOS appears to depend on the presence of the enzyme cofactors tetrahydrobiopterin and heme, as well as the substrate L-arginine (5). The nNOS homodimer is also stabilized by tetrahydrobiopterin and L-arginine (6). Since cellular levels of cofactors and substrate may themselves be regulated, enzyme oligomerization may provide a mechanism for modulation of NOS activity.

In contrast to the other NOS isoforms, which can be studied as soluble dimeric enzymes, ecNOS is predominantly membrane-associated (9), a feature that has hampered direct hydrodynamic analyses of its oligomeric structure. In the present study, we use co-immunoprecipitation experiments to show that ecNOS is an oligomeric protein and that both N- and C-terminal domains of the enzyme appear to be involved in its assembly. Furthermore, these studies document that co-expression of ecNOS truncation mutants with the wild-type enzyme exerts a dominant negative effect on enzyme activity.

### MATERIALS AND METHODS

**Plasmid Constructs**—Constructs encoding wild-type ecNOS or the myristoylation-deficient mutant (myr<sup>-</sup>), as well as their hemagglutinin (HA) epitope-tagged counterparts, have previously been described (10, 11). To prepare epitope-tagged truncation mutants, the sequence for the HA epitope followed by a stop codon was first subcloned into the pBK-CMV expression vector to form pK-HA (11). Truncation mutants of ecNOS lacking either the N- or C-terminal domains were then created using propitious restriction enzyme sites within the ecNOS sequence; these constructs are shown schematically in Fig. 1. To delete the C terminus, the ecNOS cDNA was digested with *EcoRI* and *KpnI*, yielding a fragment that contains the first 2226 base pairs and encodes the N-terminal 736 amino acids (residues 1–736). This fragment was subcloned into pK-HA in frame with the epitope tag. The resulting construct, pΔKpn, is predicted to yield a protein of  $M_r = 82$  kDa. To delete the N-terminal half of the protein, the plasmid encoding epitope-tagged ecNOS was digested with *BglII* and *BamHI* to remove the 5' 1536 bases of the ecNOS cDNA. The restricted plasmid was ligated and cloned to yield the construct pKCT encoding the C-terminal 694 amino acids of ecNOS. The first methionine codon within this truncated construct (methionine 512 in ecNOS) lies within a sequence favorable for translation initiation and would direct the expression of the C-terminal ~½ of ecNOS plus the HA epitope. Expression of pKCT is predicted to yield a protein of  $M_r = 77.6$  kDa.

**Cell Culture, Transfection, and Biosynthetic Labeling**—COS-7 cells were maintained and transfected in 100-mm cell culture dishes (using the DEAE-dextran method) as described previously (12). Biosynthetic labeling of transfected cells with [<sup>3</sup>H]myristic acid (Amersham Corp.) or [<sup>35</sup>S]methionine (Tran<sup>35</sup>S-label, ICN) was performed exactly as before (10, 13).

**NOS Enzymatic Assays**—In transfected cells, NO synthase activity

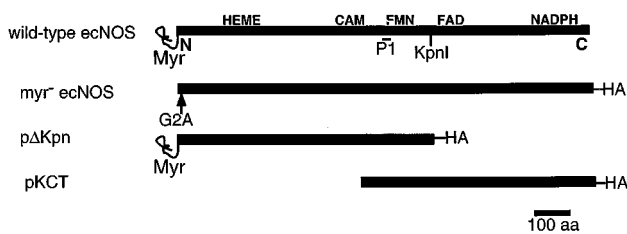
\* These studies were supported in part by National Institutes of Health Grants HL46457 and HL09172. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Fellow of the Sarnoff Foundation for Cardiovascular Research.

§ Recipient of an Individual National Research Service Award from the National Institutes of Health.

¶ Wyeth-Ayerst Established Investigator of the American Heart Association. To whom correspondence should be addressed: Cardiovascular Division, Brigham and Women's Hospital, Harvard Medical School, 75 Francis St., Boston, MA 02115. Tel.: 617-732-7376; Fax: 617-732-5132; E-mail: Michel@Calvin.BWH.harvard.edu.

<sup>1</sup> The abbreviations used are: NOS, nitric oxide synthase; NO, nitric oxide; ecNOS, iNOS, nNOS, endothelial, inducible and neuronal isoforms (respectively) of nitric oxide synthase; myr<sup>-</sup> ecNOS, myristoylation-deficient mutant of ecNOS; HA, influenza hemagglutinin epitope; PAGE, polyacrylamide gel electrophoresis; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.



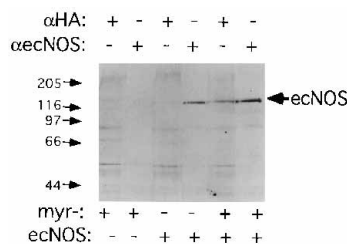
**FIG. 1. Schematic of ecNOS constructs used in co-transfection experiments.** cDNA encoding the full-length wild-type ecNOS (135 kDa) was cleaved at propitious restriction enzyme sites to yield two truncation mutants, each cloned such that the 3'-end is joined to the sequence for the HA epitope tag followed by a stop codon. The protein encoded by the mutant cDNA pΔKpn contains residues 1–734 of ecNOS. The pKCT truncation mutant encodes residues 512–1204 of ecNOS. The site for mutagenesis (*G2A*) in the myristoylation consensus sequence is shown (*myr*<sup>-</sup>); this mutation results in formation of acylation-deficient ecNOS, which was epitope-tagged at the 3'-end of the construct. Also noted are the putative sites for heme, calmodulin (*CAM*), FMN, FAD, and NADPH binding to ecNOS, as well as the location (*P1*) of the ecNOS peptide sequence (residues 597–612) used as an epitope for generation of ecNOS antiserum. *aa*, amino acids.

was assayed in cell lysates by measuring the conversion of L-[<sup>3</sup>H]arginine to L-[<sup>3</sup>H]citrulline, as described previously (10, 12). NADPH diaphorase activity was determined by staining of fixed cells, as before (12).

**Immunoprecipitation**—COS-7 cells were harvested by scraping in phosphate-buffered saline, pelleted by centrifugation, and then resuspended and sonicated in a hypotonic buffer containing 50 mM Tris (pH 7.4), 0.1 mM EGTA, 0.1 mM EDTA, 2.1 mM β-mercaptoethanol, and protease inhibitors (1 μg/ml each of soybean trypsin inhibitor, lima bean trypsin inhibitor, leupeptin, and antipain) to yield the cell lysate. Immunoprecipitation conditions were modified from those previously described (10, 11, 13). A non-denaturing detergent was used to solubilize the enzyme to better preserve the native structure of ecNOS. The cell lysates were diluted in 4 volumes of CHAPS buffer containing 20 mM CHAPS (Sigma), 50 mM Tris-HCl (pH 7.4), 0.1 mM EDTA, 0.1 mM EGTA, 2 mM dithiothreitol, 125 mM NaCl, 4 μM tetrahydrobiopterin (Schircks Laboratories), 1 mM L-arginine, and protease inhibitors as above. Duplicate aliquots of cell lysates were incubated either with a previously characterized (10) antipeptide antiserum raised against ecNOS, at a final dilution of 1:100, or with the 12CA5 monoclonal antibody for the HA epitope tag (Boehringer Mannheim), at a final concentration of 10 μg/ml. After 1 h at 4 °C, protein A-Sepharose beads (Pharmacia Biotech Inc.) were added to the samples. After a further incubation for 1 h at 4 °C, bound immune complexes were washed three times with CHAPS buffer and then once with 150 mM NaCl, 50 mM Tris (pH 7.4) supplemented with 4 μM tetrahydrobiopterin and 1 mM L-arginine. Immunoprecipitated proteins were eluted from the protein A-Sepharose beads by boiling for 5 min in SDS-PAGE sample buffer and then analyzed by SDS-PAGE followed by fluorography (En<sup>3</sup>Hance, DuPont NEN). The gels were exposed to x-ray film at -70 °C using intensifying screens.

## RESULTS AND DISCUSSION

**Co-immunoprecipitation of Wild-type and *myr*<sup>-</sup> ecNOS**—To explore whether ecNOS forms oligomers, we constructed cDNAs encoding several epitope-tagged ecNOS mutants (Fig. 1) and investigated the association of mutant and wild-type proteins by co-immunoprecipitation from transfected cells. COS-7 cells were transfected with cDNAs for wild-type ecNOS or an epitope-tagged myristoylation-deficient mutant (*myr*<sup>-</sup>) and biosynthetically labeled with [<sup>3</sup>H]myristic acid (Fig. 2). Duplicate samples of cell lysates were immunoprecipitated with either the anti-ecNOS antibody or the antibody to the epitope tag. As previously observed, the point mutation in the *myr*<sup>-</sup> mutant prevents the incorporation of myristate (10); thus, no labeled protein is specifically immunoprecipitated from cells transfected with this construct alone (Fig. 2). Wild-type ecNOS is labeled with [<sup>3</sup>H]myristate, as previously observed (10), and when expressed alone is immunoprecipitated by the anti-ecNOS antibody but not the antibody to the HA



**FIG. 2. Co-immunoprecipitation of wild-type ecNOS with epitope-tagged *myr*<sup>-</sup> ecNOS in [<sup>3</sup>H]myristate-labeled COS-7 cells.** Shown in this figure are the results of SDS-PAGE and fluorography of wild-type and mutant ecNOS immunoprecipitated from cells transfected with cDNAs encoding the epitope-tagged, myristoylation-deficient mutant (*myr*<sup>-</sup>) or the wild-type untagged enzyme (*ecNOS*) or co-transfected with both cDNAs, as indicated below the fluorogram. The transfected cells were biosynthetically labeled with [<sup>3</sup>H]myristic acid; duplicate aliquots from each cell lysate were immunoprecipitated either with the 12CA5 monoclonal antibody directed against the HA epitope (*αHA*) or antiserum directed against ecNOS (*αecNOS*) as indicated. The immunoprecipitated proteins were resolved by SDS-PAGE and subjected to fluorography on x-ray film for 3 weeks. The molecular mass of standards is shown in kDa; the site for migration of ecNOS is noted. The results shown are representative of three independent experiments.

epitope (Fig. 2). However, when untagged ecNOS is co-transfected with the HA-tagged *myr*<sup>-</sup> mutant, a [<sup>3</sup>H]myristate-labeled protein corresponding to ecNOS is immunoprecipitated by the HA antibody. This finding documents that the wild-type ecNOS associates with the tagged *myr*<sup>-</sup> mutant and thereby demonstrates that ecNOS is an oligomeric enzyme. Furthermore, since the *myr*<sup>-</sup> mutant undergoes neither myristoylation nor palmitoylation (11) and is exclusively cytosolic (10), it appears that oligomerization of ecNOS requires neither membrane association nor acylation. Our demonstration that ecNOS, like the iNOS and nNOS isoforms, is an oligomeric protein raises the possibility that it may form heteromeric complexes with these other isoforms. In cells able to express multiple isoforms of NOS, such as cardiac myocytes (14), hetero-oligomerization could provide an additional mechanism for regulating NO synthesis.

**Co-immunoprecipitation of Wild-type and Truncation Mutant ecNOS**—Having observed that ecNOS can form oligomers, we wished to define the regions of the protein involved in subunit association. The NO synthases appear to be composed of two structurally and functionally distinct domains (1, 15–19). The C-terminal or “reductase” domain shows striking sequence similarity to cytochrome P-450 reductase and contains consensus binding sequences for flavins and NADPH (18). The N-terminal domain of NOS has been termed the “heme domain” and includes the proximal thiolate heme ligand and the likely site for tetrahydrobiopterin binding (15–17, 19). We constructed two epitope-tagged truncation mutants of ecNOS: pKCT, encoding the C-terminal reductase domain, and pΔKpn, containing the N-terminal heme domain (Fig. 1). The pKCT mutant was strongly positive in the NADPH diaphorase assay, consistent with preservation of the reductase function of the C-terminal domain (Fig. 3). By contrast, the pΔKpn mutant was negative in the NADPH diaphorase assay, likely reflecting the absence of essential redox cofactor binding sites in this construct. Neither truncation mutant supported NO synthesis (see below).

We investigated which domains are involved in ecNOS oligomerization by co-expression and immunoprecipitation of wild-type ecNOS with these epitope-tagged truncation mutants. As shown in Fig. 4, the protein encoded by the pΔKpn truncation mutant can be expressed in COS cells and is immunoprecipitated with either the ecNOS antibody or the antibody to the epitope tag. Wild-type ecNOS, when expressed alone, can

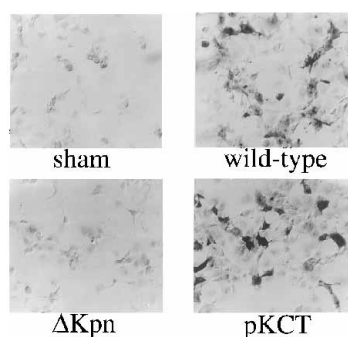


FIG. 3. NADPH diaphorase staining of wild-type and truncation mutant ecNOS transfected into COS-7 cells. Shown are photomicrographs of COS-7 cells transfected with the plasmid vector alone (*sham*), with cDNAs encoding wild-type ecNOS (*wild-type*) or truncation mutants expressing either N-terminal ( $\Delta Kpn$ ) or C-terminal (*pKCT*) domains of ecNOS (see Fig. 1). Seventy-two hours after transfection, cultures were fixed and stained with nitroblue tetrazolium to detect NADPH diaphorase activity.

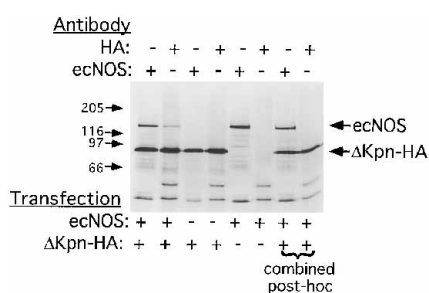


FIG. 4. Co-immunoprecipitation of full-length ecNOS and C-terminal truncation mutant. Shown is a fluorogram of the SDS-PAGE analysis of proteins immunoprecipitated from COS-7 cells transfected with cDNAs for untagged full-length ecNOS and/or an epitope-tagged truncation mutant lacking the protein's C terminus ( $\Delta Kpn$ -HA). Transfected cells were biosynthetically labeled with [ $^{35}$ S]methionine, and duplicate samples of cell lysates were immunoprecipitated using the HA antibody against the epitope tag or anti-ecNOS antiserum, as noted above the fluorogram. Minor bands reflect nonspecific immunoprecipitation of cellular proteins by the monoclonal HA antibody, as previously reported (11). In the *first two lanes* are the results of immunoprecipitations from cultures co-transfected with both the  $\Delta Kpn$  and wild-type ecNOS cDNAs. In the following lanes are proteins immunoprecipitated from cultures transfected with only one of these constructs (indicated below the fluorogram); the *last two lanes* show the pattern of immunoprecipitation seen when such lysates (containing either  $\Delta Kpn$  or wild-type ecNOS) are mixed together immediately prior to immunoprecipitation (*post-hoc*). Immunoprecipitated proteins were analyzed by SDS-PAGE and fluorography; this film was exposed for 3 days. On the right, arrows indicate bands corresponding to the full-length ecNOS (135 kDa) and the  $\Delta Kpn$ -HA truncation mutant protein (82 kDa). The molecular mass standards are shown in kDa on the left. The results shown are representative of three independent experiments.

be immunoprecipitated only by the ecNOS antibody. However, when wild-type ecNOS is co-expressed with the epitope-tagged mutant, the full-length enzyme is now immunoprecipitated by the HA antibody (Fig. 4). This suggests that the full-length protein associates with the tagged truncation mutant in a hetero-oligomeric complex. When lysates of cells transfected separately with ecNOS or  $\Delta Kpn$  were combined *in vitro*, ecNOS did not co-immunoprecipitate with the truncation mutant (Fig. 4). This result implies that the observed association of co-expressed full-length and truncated ecNOS primarily occurs intracellularly and not following cell lysis.

A similar approach was employed to examine the role of the C-terminal domain in enzyme oligomerization. COS-7 cells were transfected with constructs for wild-type ecNOS or the HA-tagged truncation mutant, pKCT (Fig. 5); as before, when expressed alone, the untagged wild-type enzyme was immunoprecipitated by the ecNOS antibody but not by the antibody to

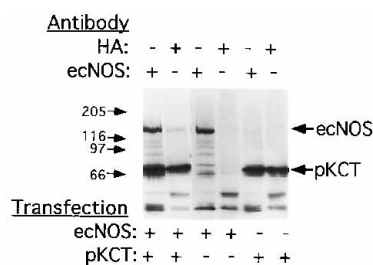
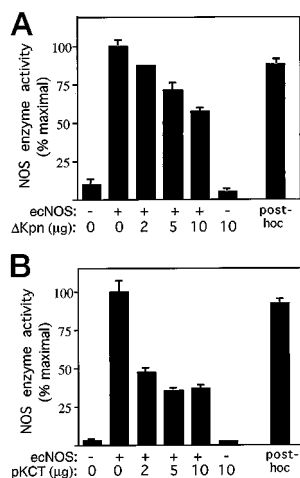


FIG. 5. Co-immunoprecipitation of full-length ecNOS and N-terminal truncation mutant. This figure shows a fluorogram of the SDS-PAGE analysis of proteins immunoprecipitated from COS-7 cells biosynthetically labeled with [ $^{35}$ S]methionine following transfection with cDNAs (indicated below the fluorogram) encoding the untagged full-length ecNOS and/or an epitope-tagged N-terminal truncation mutant of ecNOS (*pKCT*). Proteins were immunoprecipitated from duplicate samples of cell lysates using either the HA or ecNOS antibody, as noted above the fluorogram. The immunoprecipitated proteins were analyzed by SDS-PAGE and fluorography; this film was exposed for 3 days. On the right, arrows indicate bands corresponding to the full-length ecNOS (135 kDa) and the pKCT truncation mutant protein (78 kDa). The molecular weight standards are shown in kDa on the left. Results are representative of three independent experiments.

the epitope tag. Epitope-tagged pKCT could be immunoprecipitated by either antibody. When the wild-type enzyme was co-expressed with pKCT, full-length ecNOS could now be immunoprecipitated by the HA antibody, consistent with the formation of a hetero-oligomeric complex. Together these results suggest that both the N- and C-terminal domains of ecNOS may be involved in oligomerization of the enzyme. These findings may be contrasted with a recent study of iNOS oligomerization by Ghosh and Stuehr (19). In this report, limited tryptic digestion of purified iNOS (which cleaves in mid-molecule to separate N- and C-terminal domains) led to the formation of dimeric N-terminal and monomeric C-terminal peptides. Therefore, it was proposed for iNOS that only the N-terminal domain, and not the C-terminal domain, participates in dimer formation. However, our results document specific interactions between the full-length enzyme and both the N- and C-terminal domains, since the N- and C-terminal truncation mutants are each able to co-immunoprecipitate ecNOS. These divergent results may represent intrinsic differences between iNOS and ecNOS oligomerization or may reflect differences in experimental approach.

Both the N- and C-terminal ecNOS truncation mutants thus appear to form hetero-oligomeric complexes with the wild-type enzyme. However, only a fraction of the wild-type ecNOS is co-immunoprecipitated by these truncation mutants (Figs. 4 and 5). It is possible that stronger associations between full-length ecNOS monomers result in preferential formation of homo-oligomers of the wild-type enzyme. Indeed, when ecNOS is expressed with the full-length HA-tagged myr<sup>-</sup> mutant, a larger fraction of the wild-type enzyme is co-immunoprecipitated by the HA antibody (Fig. 2). Weaker interactions between truncation mutants and full-length ecNOS might also lead to a substantial dissociation of hetero-oligomeric complexes during immunoprecipitation, with consequent loss of the full-length enzyme.

**Dominant Negative Effect of Co-transfected ecNOS Truncation Mutants on NOS Activity**—To examine the effects of hetero-oligomerization on NOS enzyme activity, we measured the conversion of L-[ $^3$ H]arginine to L-[ $^3$ H]citrulline in lysates of cells transfected with ecNOS and either the C- or N-terminal truncation mutants. Essentially no NOS activity was seen in sham-transfected COS cells or in cells expressing only the  $\Delta Kpn$  (Fig. 6A) or pKCT (Fig. 6B) truncation mutants. Transfection of wild-type ecNOS cDNA resulted in substantial NOS enzyme activity, but when either truncation mutant was co-



**FIG. 6. Dominant negative effect of truncation mutants on wild-type ecNOS enzyme activity.** NOS activity was assayed in lysates of transfected cells by measuring the conversion of [<sup>3</sup>H]arginine to [<sup>3</sup>H]citrulline. The effects of co-expression of the truncation mutants pΔKpn (*panel A*) and pKCT (*panel B*) with wild-type ecNOS were analyzed in separate experiments. The activity data shown (mean ± S.D.) are expressed as a percentage of the NOS activity of the wild-type enzyme alone; for the experiment shown in *panel A*, wild-type NOS activity was 1.4 pmol of [<sup>3</sup>H]citrulline formed/min-mg protein and for *panel B* was 1.2 pmol/min-mg protein. For each transfection, the total amount of DNA transfected is kept constant by the addition of vector DNA. Duplicate aliquots of the transfected cell lysates were also analyzed by protein immunoblot (probed with ecNOS antiserum); the transfection of equal quantities of wild-type ecNOS cDNA yielded similar levels of protein expression (data not shown). In both *panels A* and *B*, the *first column* represents NOS activity in COS cells transfected with vector alone (sham) and the *next column* the activity of the wild-type enzyme (5 μg of cDNA/culture) expressed alone. The *following three columns* show the results of co-expressing the same quantity of wild-type cDNA with 2, 5, or 10 μg of truncation mutant cDNA, as noted below the histogram; the *next column* represents activity in COS cells transfected with only the truncation mutant (5 μg of DNA/culture). The *last column* represents activity measured when cells are transfected separately with either wild-type or truncated ecNOS cDNAs, and the lysates, containing either wild-type or mutant ecNOS, are subsequently combined *in vitro* just prior to the activity assay (*post-hoc*). The experiments shown are representative of three similar experiments, each conducted in triplicate.

expressed, enzyme activity was diminished, documenting a dominant negative effect of these mutants. Furthermore, the attenuation in NOS activity increased as the amount of the co-transfected mutant cDNA was increased (total DNA in each transfection was kept constant by the addition of vector DNA). The degree of inhibition observed with co-transfection could not be reproduced by combining wild-type and mutant ecNOS *in*

*vitro* (Fig. 6), conditions under which no hetero-oligomerization could be detected by co-immunoprecipitation (Fig. 4). The dominant negative effects of ecNOS truncation mutants may thus reflect the intracellular formation of inactive hetero-oligomers.

Dimerization has been shown to be necessary for the catalytic activity of other isoforms of NOS and may be modulated by levels of substrates and cofactors (5, 6). It seems likely that ecNOS oligomerization is also required for catalysis, most plausibly involving formation of an intact ecNOS homodimer. The dominant negative effects of ecNOS truncation mutants may reflect their competition with full-length monomers for the same sites of association. These results also suggest that intersubunit interactions are involved in the complex redox pathway from arginine to citrulline plus NO. These speculations will be refined by studies of the structural features of hetero-oligomeric ecNOS complexes; such studies are likely to provide important insights into the mechanisms underlying the dominant negative effect of the truncation mutants. Characterization of the regions involved in NOS dimerization may lead to the identification of a new class of enzyme inhibitors that exert a dominant negative effect on NOS activity by inhibiting enzyme oligomerization.

*Acknowledgment*—We are grateful to Dr. Kazuhiro Sase for helpful discussions.

#### REFERENCES

- Lowenstein, C. J., and Snyder, S. H. (1992) *Cell* **70**, 705–707
- Stuehr, D. J., and Griffith, O. W. (1992) *Adv. Enzymol. Relat. Areas Mol. Biol.* **65**, 287–346
- Nathan, C., and Xie, Q.-W. (1994) *J. Biol. Chem.* **269**, 13725–13728
- Marletta, M. (1993) *J. Biol. Chem.* **268**, 12231–12234
- Baek, K. J., Thiel, B. A., Lucas, S., and Stuehr, D. J. (1993) *J. Biol. Chem.* **268**, 2120–2129
- Klatt, P., Schmidt, K., Lehner, D., Glatter, O., Bächinger, H. P., and Mayer, B. (1995) *EMBO J.* **14**, 3687–3695
- Austin, A. J., Crabtree, G. R., and Schreiber, S. L. (1994) *Chem. Biol.* **1**, 131–136
- Heldin, C.-H. (1995) *Cell* **80**, 213–223
- Pollock, J. S., Forstermann, U., Mitchell, J. A., Warner, T. D., Schmidt, H. W., Nakane, M., and Murad, F. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88**, 10480–10484
- Busconi, L., and Michel, T. (1993) *J. Biol. Chem.* **268**, 8410–8413
- Robinson, L. J., Busconi, L., and Michel, T. (1995) *J. Biol. Chem.* **270**, 995–998
- Lamas, S., Marsden, P. A., Li, G. K., Tempst, P., and Michel, T. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89**, 6348–6352
- Michel, T., Li, G. K., and Busconi, L. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90**, 6252–6256
- Balligand, J.-L., Kobzik, L., Han, X., Kaye, D. M., Belhassen, L., O'Hara, D. S., Kelly, R. A., Smith, T. W., and Michel, T. (1995) *J. Biol. Chem.* **270**, 14582–14586
- Richards, M., and Marletta, M. A. (1994) *Biochemistry* **33**, 14723–14732
- McMillan, K., and Masters, B. S. S. (1995) *Biochemistry* **34**, 3686–3693
- Sheta, E. A., McMillan, K., and Masters, B. S. S. (1994) *J. Biol. Chem.* **269**, 15147–15153
- Bredt, D. S., Hwang, P. M., Glatt, C. E., Lowenstein, C., Reed, R. R., and Snyder, S. H. (1991) *Nature* **351**, 714–718
- Ghosh, D. K., and Stuehr, D. J. (1995) *Biochemistry* **34**, 801–807