0026-895X/95/040655-05$3.00/0

MOLECULAR PHARMACOLOGY, 47:655–659 (1995).

ACCELERATED COMMUNICATION

Recombinant Endothelial Nitric Oxide Synthase: Post-translational Modifications in a Baculovirus Expression System

LILIANA BUSCONI[1] and THOMAS MICHEL

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

Received December 27, 1994; Accepted January 23, 1995

SUMMARY

Nitric oxide synthesized by the endothelial isoform of nitric oxide synthase (ecNOS) is importantly involved in the homeostatic control of blood pressure and platelet aggregation. The different members of the nitric oxide synthase protein family have several biochemical features in common but serve distinct physiological functions and are the products of distinct genes. The ecNOS is further distinguished by its subcellular distribution in the endothelial cell membrane, and the enzyme undergoes several post-translational modifications, including myristoylation, palmitoylation, and phosphorylation. Overall, however, the ecNOS has remained less well characterized because of the challenges involved in isolating sufficient quantities of this membrane-associated protein from native or cultured endothelial cells. In this report, we describe the purification and characterization of ecNOS expressed in a heterologous system in recombinant baculovirus-infected insect Sf9 cells. Recombinant ecNOS is targeted to the Sf9 cell membrane and comprises ~10% of the total cellular protein, allowing purification to homogeneity in a single-step procedure to yield a stable protein that retains the essential features of the native enzyme. Using biosynthetic labeling and immunoprecipitation, we show that recombinant ecNOS is myristoylated, palmitoylated, and phosphorylated when expressed in insect Sf9 cells. The interpretation of structural and enzymological studies of recombinant ecNOS will be facilitated by the apparent fidelity of its biosynthesis and post-translational modification in insect Sf9 cells.

Nitric oxide is the active metabolite of organic nitrate vasodilator drugs and is synthesized in the body by a family of NOS enzymes (for reviews, see Refs. 1–3). These NOS enzymes catalyze the oxidation of L-arginine to form nitric oxide plus L-citrulline, in a complex reaction scheme involving numerous redox cofactors. The three mammalian NOS isoforms characterized to date all appear to share similar overall mechanisms for the oxidation of L-arginine to form nitric oxide, although important regulatory differences have been noted (4, 5). Despite their common enzymological and structural features, the different NOS isoforms are involved in biological processes as diverse as neurotransmission (neural or type I NOS), cytotoxicity (inducible or type II NOS), and blood pressure homeostasis (ecNOS or type III NOS). These three NOS isoforms are the products of distinct genes located on different human chromosomes and show distinct tissue-specific patterns of expression (6).

The ecNOS is targeted to the particulate subcellular fraction in endothelial cells (7, 8), in contrast to the other NOS isoforms, which are principally found in cell cytosol (3). Targeting of ecNOS to endothelial membranes is dependent upon two distinct covalent modifications, i.e., the co-translational addition of the 14-carbon fatty acid myristic acid to an amino-terminal glycine residue (9) and the post-translational addition of the 16-carbon fatty acid palmitic acid to a cysteine residue (10). However, the subcellular localization of ecNOS is dynamically regulated, in that agonist treatment of endothelial cells results in the translocation of the enzyme from membrane to cytosol (11), which is apparently caused by agonist-induced depalmitoylation of ecNOS; phosphorylation of the enzyme then occurs in the endothelial cell cytosol (10).

This work was supported by funds from the National Institutes of Health (Grant HL46457) and the American Heart Association. T.M. is a Wyeth-Ayerst Established Investigator of the American Heart Association.

[1] Current address: Renal Division, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115.

ABBREVIATIONS: NOS, nitric oxide synthase; ecNOS, endothelial cell nitric oxide synthase; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate.

The post-translational modifications of ecNOS thus importantly affect its subcellular localization and may be important points of regulation *in situ*.

A more detailed understanding of the enzymological and structural features of ecNOS has lagged behind studies of the other NOS isoforms, due in no small part to the challenges of purifying the membrane-associated ecNOS from native or cultured endothelial cells. However, any heterologous system for the expression of ecNOS must take into account its complex post-translational modifications, to validate the features of the native enzyme. In this report, we describe the expression, purification, and characterization of ecNOS in insect Sf9 cells infected with a recombinant baculovirus encoding ecNOS cDNA. Recombinant ecNOS can be purified in a single step from infected Sf9 cells, and the enzyme appears to retain the essential biosynthetic and biochemical features of the native enzyme.

Materials and Methods

Construction of recombinant transfer vector and recombinant virus. The ecNOS plasmid (12) was digested with *Xba*I and *Eco*RV, and the cDNA insert was subcloned into the baculovirus transfer vector pVL1393 (Pharmingen), which had been previously digested with *Sma*I and *Xba*I. Recombinant virus containing the ecNOS cDNA was generated by homologous recombination between the transfer vector and linearized BaculoGold (Pharmingen) virus DNA, according to the manufacturer's instructions. Plaque-purified recombinant virus expressing ecNOS was termed BV-ecNOS.

Cell culture and viral infection. Insect Sf9 cells were grown in Grace's supplemented insect medium (GIBCO) containing 10% fetal bovine serum (Hyclone). Cells were maintained in suspension culture and infected in monolayer culture as described (13). For a typical infection with recombinant virus, one 150-cm^2 tissue culture flask was seeded with 1.8×10^7 exponentially growing Sf9 cells in 20 ml of medium and then infected with the BV-ecNOS virus at a multiplicity of infection of 5–10. At varying times after infection (generally 72 hr), the cells were harvested and then pelleted by centrifugation at 1000 × *g* for 5 min.

NADPH diaphorase assay. Insect Sf9 cells were stained for NOS activity using the NADPH diaphorase assay (14, 15).

Purification of ecNOS. The purification of recombinant ecNOS was modified from a protocol for the purification of the native enzyme (8). The BV-ecNOS-infected Sf9 cell pellet from one 150-cm^2 tissue culture flask was resuspended in 6 ml of buffer 1 [50 mM Tris·HCl, pH 7.4, 0.1 mM EGTA, 0.1 mM EDTA, 2 mM 2-mercaptoethanol, 150 mM NaCl, plus protease inhibitors (9)] and sonicated. CHAPS and $CaCl_2$ were added to final concentrations of 1.25% and 1.2 mM, respectively, the suspension was rotated for 30 min at 4° to solubilize cell membranes, and the remaining particulate material was pelleted by centrifugation at 12,000 × *g* for 15 min. Glycerol was added (to a final concentration of 10%) to the supernatant fraction, which was then incubated with preswollen calmodulin-agarose [prepared from a 1:2, v/v, mixture of calmodulin-agarose (Sigma) and Sepharose 4B]. The suspension was gently mixed for 45 min at 4°, transferred to a fritted chromatography column, and washed with 10 volumes of buffer 2 (buffer 1 plus 10% glycerol, 1.25% CHAPS, and 1.2 mM $CaCl_2$). ecNOS was then eluted with buffer 3 (buffer 1 plus 10% glycerol, 1.25% CHAPS, and 5 mM EDTA).

Protein determination. Protein concentration was determined using a modified Bradford protein assay, according to the manufacturer's instructions (Bio-Rad), using bovine serum albumin as a standard.

Assay of NOS activity. NOS activity was assayed by the conversion of L-[^{3}H]arginine (DuPont-NEN) to L-[^{3}H]citrulline, using conditions essentially identical to those described previously (9), with the exception that FAD and FMN (1 μM final concentrations) were added to the assay mixture.

Biosynthetic labeling, subcellular fractionation, and immunoprecipitation. Biosynthetic labeling of insect Sf9 cells was modified only slightly from our previously described protocols (9–11). Grace's insect medium was used for labeling cells with [^{35}S]methionine (NEN-DuPont) (50 μCi/ml, for 4 hr) or with [^{3}H]palmitic acid (500 μCi/ml, for 2 hr); Ex-Cell 401 modified phosphate-free medium (JRH Biosciences) was used for labeling with [^{32}P]orthophosphate (NEN-DuPont) (100 μCi/ml, for 4 hr). When [^{3}H]palmitate-labeled samples were eluted for SDS-PAGE, the sample buffer contained 5 mM dithiothreitol (rather than 5% 2-mercaptoethanol), to avoid cleavage of palmitoyl thioesters.

Results and Discussion

Expression and purification of recombinant ecNOS. Infection of insect Sf9 cells with the BV-ecNOS recombinant baculovirus yields a robust pattern of enzyme expression. Analysis of BV-ecNOS-infected Sf9 cells using the NADPH diaphorase assay shows strong staining, relative to uninfected cells (Fig. 1A). This staining is specifically due to expression of ecNOS, as confirmed by the results (Fig. 1B) of biosynthetic [^{35}S]methionine labeling and immunoprecipitation of the enzyme from infected but not uninfected cells,

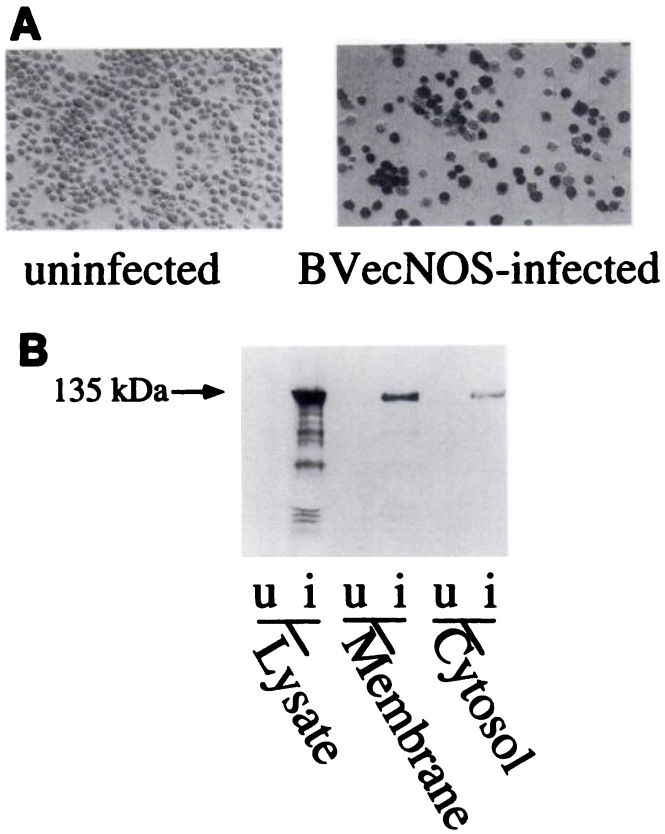

Fig. 1. Patterns of ecNOS expression in insect Sf9 cells infected with recombinant baculovirus BV-ecNOS. A, Representative photomicrographs of insect Sf9 cells that were uninfected (*left*) or infected (*right*) with BV-ecNOS and analyzed 72 hr after infection, using the NADPH diaphorase assay, as described in the text. B, Fluorogram of SDS-PAGE analysis of [^{35}S]methionine-labeled proteins from insect Sf9 cell that were uninfected (*u*) or infected (*i*) with BV-ecNOS; proteins were immunoprecipitated with ecNOS-specific antiserum from lysate or from cell cytosol or membrane fractions resolved by ultracentrifugation. This experiment was replicated four times, with equivalent results.

using a highly ecNOS-specific antipeptide antiserum we have extensively characterized (9). The recombinant protein expressed in BV-ecNOS-infected cells is found primarily in the particulate subcellular fraction (Fig. 1B), as has been previously observed for the native enzyme in endothelial cells. In infected cells, ecNOS expression accounts for ~10% of total cellular protein, with maximum levels of expression being reached 48–72 hr after infection, to the point that ecNOS protein is easily visualized in Coomassie blue-stained gels (Fig. 2A). The membrane-associated ecNOS was solubilized in the detergent CHAPS and purified ~6-fold, to homogeneity, in a one-step protocol using affinity chromatography on a calmodulin-agarose column, with a 20% yield (Fig. 2B; Table 1). The purified enzyme is stable for several weeks when stored in buffer at −20° in 50% glycerol; under similar conditions but with 10% glycerol, enzyme activity is not maintained. The purified recombinant enzyme shows the same overall cofactor requirements as does native ecNOS, and its specific activity of 7.3 nmol/min/mg of protein is similar to that reported previously for the native enzyme (8).

These results document that high levels of ecNOS protein expression are achieved in BV-ecNOS-infected insect Sf9 cells and the recombinant ecNOS can be purified to homogeneity in a single chromatographic step. The purified recombinant enzyme is stable and has enzymological features like those established for native ecNOS. As has been previously documented for ecNOS expressed in vascular endothelial cells, we found that the recombinant ecNOS is present primarily in the particulate subcellular fraction in infected insect Sf9 cells.

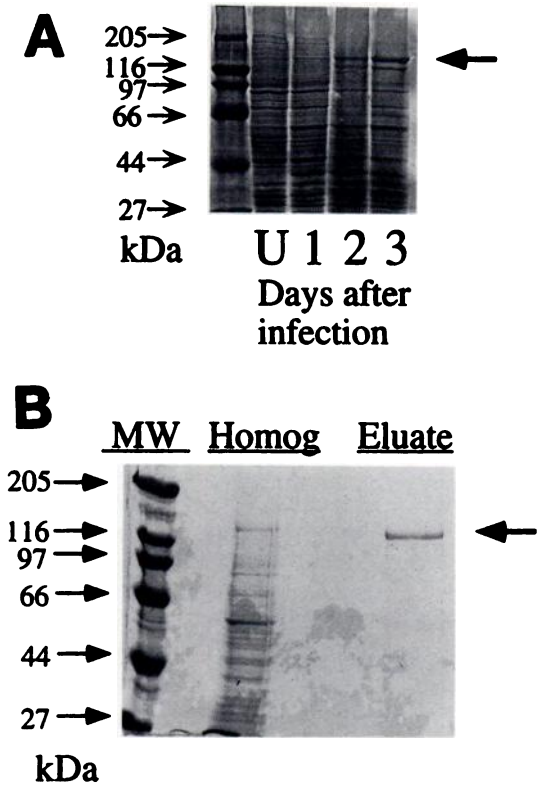

Fig. 2. Expression and purification of ecNOS from BV-ecNOS-infected insect Sf9 cells. A, Pattern of cellular protein expression in insect Sf9 cells harvested at the indicated times after infection with BV-ecNOS and analyzed by SDS-PAGE and staining with Coomassie blue. The control cells uninfected (*U*) with BV-ecNOS were harvested 3 days after sham infection. *Leftmost lane*, molecular weight markers. The gel shown represents 25% of the total cellular extract obtained from infection of 1.8×10^7 cells (grown in 20 ml of culture medium in a 150-cm^2 culture flask) with recombinant BV-ecNOS baculovirus, at a multiplicity of infection of 5. B, Representative single-step purification of ecNOS from BV-ecNOS-infected insect Sf9 cells, analyzed by SDS-PAGE and staining with Coomassie blue. Three days after infection, the cells were harvested and solubilized in CHAPS (*Homog*), chromatographed on calmodulin-agarose, and eluted with EGTA-containing buffer (*Eluate*) as described in the text. *MW*, molecular weight markers.

TABLE 1

Purification of ecNOS from BV-ecNOS-infected insect Sf9 cells

Insect Sf9 cells (~10^7 in one 150-cm^2 culture flask) were infected with BV-ecNOS and were harvested 3 days later. The infected insect cells were sonicated and solubilized with CHAPS-containing buffer, and the soluble extract was chromatographed over calmodulin-agarose and eluted with EGTA-containing buffer as described in the text. NOS enzyme activity was evaluated by measuring the conversion of L-[^{3}H]arginine to [^{3}H]citrulline. This purification protocol was repeated three times, with similar results.

Fraction	Volume	Protein	Specific Activity	Yield
	ml	*mg*	*nmol/mg/min*	*%*
Solubilized homogenate	6	6	1.2	100
Calmodulin-agarose eluate	10	0.23	7.3	23

Post-translational modifications of recombinant ecNOS. Detailed structural or enzymological analyses of recombinant ecNOS must proceed with some assurance that the enzyme has undergone appropriate post-translational modifications in any heterologous expression system used. In endothelial cells, ecNOS undergoes several post-translational modifications, including myristoylation (9, 16), phosphorylation (11), and palmitoylation (10). To explore whether recombinant ecNOS undergoes similar modifications, we performed biosynthetic labeling experiments in BV-ecNOS-infected Sf9 cells and immunoprecipitated the enzyme with ecNOS-specific antiserum (9). Fig. 3 shows the result of biosynthetic labeling with [^{3}H]myristic acid and clearly documents that recombinant ecNOS is myristoylated and most of the myristoylated protein is localized to the particulate sub-

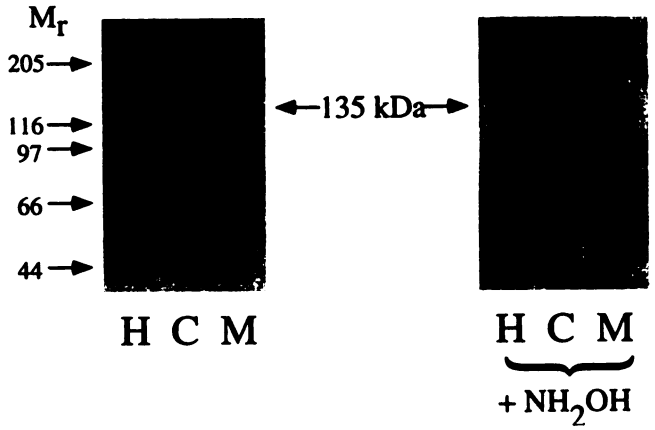

Fig. 3. Biosynthetic labeling of BV-ecNOS-infected Sf9 cells with [^{3}H]myristic acid. Two days after infection with BV-ecNOS, insect Sf9 cells were labeled for 16 hr with [^{3}H]myristic acid and harvested; the cellular homogenate (*H*) was separated into cytosolic (*C*) and membrane (*M*) fractions by ultracentrifugation and then immunoprecipitated as described in the text. After SDS-PAGE of duplicate samples, the fixed gel was treated either with 1 M Tris·HCl, pH 7, or with 1 M hydroxylamine, pH 7 (+ *NH_2OH*), for 1 hr and then processed for fluorography. The gels shown were exposed for 1 week. This experiment was replicated twice, with equivalent results. *Arrows on right* show position of the 135 kDa ecNOS protein.

cellular fraction. The labeling of ecNOS with [^{3}H]myristic acid is not sensitive to hydroxylamine, consistent with formation of a myristoyl fatty acyl amide linkage, as previously established for the native ecNOS. Biosynthetic labeling of BV-ecNOS-infected cells with [^{3}H]palmitic acid shows that the recombinant enzyme is also palmitoylated (Fig. 4A); as we have previously shown, essentially all of the palmitoylated protein is in the particulate fraction. In contrast to the results for [^{3}H]myristic acid labeling (Fig. 3), hydroxylamine treatment of [^{3}H]palmitic acid-labeled recombinant ecNOS results in loss of label (Fig. 4B), consistent with formation of a palmitoyl fatty acyl thioester with ecNOS (10). Biosynthetic labeling of BV-ecNOS-infected cells with $^{32}P_i$ (Fig. 5) shows that the recombinant ecNOS is phosphorylated; there was no substantial change in phosphorylation seen with the calcium ionophore A23187. Importantly, most of the phosphorylated ecNOS is located in the cell cytosol, in inverse relationship to the subcellular distribution of total protein.

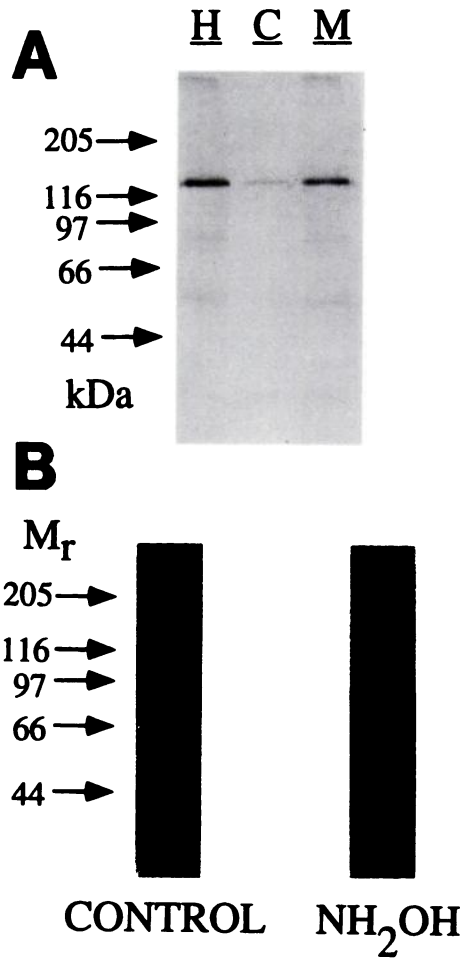

Fig. 4. Biosynthetic labeling of BV-ecNOS-infected Sf9 cells with [^{3}H]palmitic acid. A, Three days after infection with BV-ecNOS, insect Sf9 cells were labeled for 2 hr with [^{3}H]palmitic acid and then harvested; the cellular homogenate (*H*) was separated into cytosolic (*C*) and membrane (*M*) fractions by ultracentrifugation, immunoprecipitated, and analyzed by SDS-PAGE and fluorography as described in the text. The gel shown in this experiment was exposed for 1 month; these results were replicated in a separate experiment with equivalent results. B, After immunoprecipitation and SDS-PAGE of ecNOS from cell homogenates after biosynthetic labeling of infected Sf9 cells with [^{3}H]palmitic acid, duplicate samples were analyzed by treatment of the fixed gels either with 1 M Tris·HCl, pH 7 (*CONTROL*), or with 1 M hydroxylamine, pH 7 (*NH_2OH*), for 1 hr and were then processed for fluorography. The gel shown was exposed for 1 month. This experiment was replicated once, with equivalent results.

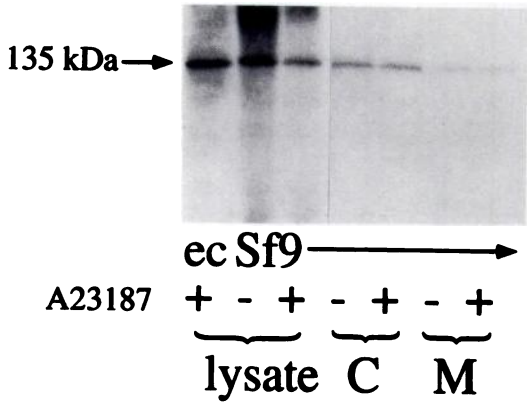

Fig. 5. Biosynthetic labeling of BV-ecNOS-infected Sf9 cells with $^{32}P_i$. BV-ecNOS-infected insect cells were biosynthetically labeled with $^{32}P_i$ as described in the text, treated (+) or not (−) with calcium ionophore A23187 (10 μM) for 5 min, harvested, lysed, and ultracentrifuged to yield cytosol (*C*) and membrane (*M*) fractions, which were immunoprecipitated with ecNOS-specific antiserum. *Leftmost lane*, ecNOS immunoprecipitated from biosynthetically $^{32}P_i$-labeled bovine aortic endothelial cells (*ec*) (11).

In vascular endothelial cells, with agonist stimulation ecNOS is translocated from the membrane to the cytosol, a process that is associated with an increase in enzyme phosphorylation (11). We have recently reported that phosphorylation of ecNOS takes place in the cell cytosol and is unlikely to be a cause of enzyme translocation. Instead, it appears that agonist-promoted depalmitoylation of ecNOS is the proximate cause of enzyme translocation from membrane to cytosol, and phosphorylation is thus consequent to rather than causal for subcellular translocation (10). Although it is clear that ecNOS phosphorylation takes place on a specific serine residue in cultured bovine aortic endothelial cells, the site of phosphorylation has yet to be definitively identified and the biological role of ecNOS phosphorylation remains obscure. As seen for the native ecNOS expressed in endothelial cells, recombinant ecNOS is phosphorylated in insect Sf9 cells and, moreover, the phosphorylated enzyme is predominantly in the cell cytosol. These similarities suggest that the baculovirus expression system might be appropriately used to study ecNOS phosphorylation, although this can be definitively approached only after the phosphorylation site is more fully characterized in endothelial cells.

These biosynthetic labeling experiments with the recombinant ecNOS also show that the enzyme is both myristoylated and palmitoylated in insect Sf9 cells, as we have previously observed for the native enzyme. The myristoylation site for native ecNOS has been identified (12) and is consistent with the myristoylation consensus sequences established for several other acylated proteins (17). Indeed, protein myristoyl transferases have been characterized, and a general consensus sequence for protein myristoylation has been described that appears to function appropriately in insect Sf9 cells (18). In contrast, the determinants of protein palmitoylation are less clearly understood. No general consensus sequence for palmitoylation has been identified (19–22), although some dually acylated proteins (e.g., G protein α subunits) (23–25) are palmitoylated at a cysteine residue within a conserved

amino-terminal sequence (MGCXXS). However, this cysteine-containing consensus sequence is not found in ecNOS, and the precise cysteine residue(s) that undergo palmitoylation in ecNOS remain obscure. Our finding that the recombinant ecNOS is palmitoylated in Sf9 cells argues strongly against the possibility of an ecNOS-specific palmitoyl transferase in endothelial cells. Indeed, enzymatic pathways in insect Sf9 cells appear to support palmitoylation of other recombinant proteins that undergo palmitoylation in their cells of origin (26, 27). Characterization of the palmitoylated recombinant ecNOS expressed in insect Sf9 cells may lead to additional insights into the role of reversible palmitoylation in the regulation of ecNOS function. The apparent fidelity of native post-translational modifications of ecNOS expressed in insect Sf9 cells indicates that enzymological and structural studies of recombinant ecNOS may proceed with reasonable confidence that the intrinsic biosynthetic and biochemical features of the native enzyme have been maintained in this heterologous expression system.

References

1. Moncada, S., R. M. J. Palmer, and E. A. Higgs. Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol. Rev.* **43:**109–142 (1991).
2. Marletta, M. A. Nitric oxide synthase: aspects concerning structure and catalysis. *Cell* **78:**927–930 (1994).
3. Nathan, C. Nitric oxide as a secretory product of mammalian cells. *FASEB J.* **6:**3051–3064 (1992).
4. Cho, H. J., Q.-w. Xie, J. Calaycay, R. A. Mumford, K. M. Swiderek, T. D. Lee, and C. Nathan. Calmodulin is a subunit of nitric oxide synthase from macrophages. *J. Exp. Med.* **176:**599–604 (1992).
5. Abu-Soud, H. M., and D. J. Stuehr. Nitric oxide synthases reveal a role for calmodulin in controlling electron transfer. *Proc. Natl. Acad. Sci. USA* **90:**10769–10772 (1990).
6. Nathan, C., and Q.-w. Xie. Regulation of biosynthesis of nitric oxide. *J. Biol. Chem.* **269:**13725–13728 (1994).
7. Boje, K. M., and H. Fung. Endothelial nitric oxide generating enzyme(s) in the bovine aorta: subcellular location and metabolic characterization. *J. Pharmacol. Exp. Ther.* **253:**20–26 (1990).
8. Pollock, J. S., U. Forstermann, J. A. Mitchell, T. D. Warner, H. H. Schmidt, M. Nakane, and F. Murad. Purification and characterization of particulate endothelium-derived relaxing factor synthase from cultured and native bovine aortic endothelial cells. *Proc. Natl. Acad. Sci. USA* **88:**10480–10484 (1991).
9. Busconi, L., and T. Michel. Endothelial nitric oxide synthase: N-terminal myristoylation determines subcellular localization. *J. Biol. Chem.* **268:** 8410–8413 (1993).
10. Robinson, L. J., L. Busconi, and T. Michel. Agonist-modulated palmitoylation of endothelial nitric oxide synthase. *J. Biol. Chem.* **270:**1–4 (1994).
11. Michel, T., G. K. Li, and L. Busconi. Phosphorylation and subcellular translocation of endothelial nitric oxide synthase. *Proc. Natl. Acad. Sci. USA* **90:**6252–6256 (1993).
12. Lamas, S., P. A. Marsden, G. K. Li, P. Tempst, and T. Michel. Endothelial nitric oxide synthase: molecular cloning and characterization of a distinct constitutive enzyme isoform. *Proc. Natl. Acad. Sci. USA* **89:**6348–6352 (1992).
13. Ausubel, F. M., R. Brent, R. E. Kingston, D. E. Moore, J. G. Seidman, J. A. Smith, and K. Struhl, eds. *Current Protocols in Molecular Biology*, Unit 16.8. Wiley, New York (1991).
14. Scherer-Singler, U., S. R. Vincent, H. Kimura, and E. G. McGeer. Demonstration of a unique population of neurons with NADPH-diaphorase histochemistry. *J. Neurosci. Methods* **9:**229–234 (1983).
15. Dawson, T. M., D. S. Bredt, M. Fotuhi, P. M. Hwang, and S. H. Snyder. Nitric oxide synthase and neuronal NADPH diaphorase are identical in brain and peripheral tissues. *Proc. Natl. Acad. Sci. USA* **88:**7797–7801 (1991).
16. Busconi, L., and T. Michel. Endothelial nitric oxide synthase membrane targeting. *J. Biol. Chem.* **269:**25016–25020 (1994).
17. Towler, D. A., J. I. Gordon, S. P. Adams, and L. Glaser. The biology and enzymology of eukaryotic protein acylation. *Annu. Rev. Biochem.* **57:**66–99 (1988).
18. Linder, M. E., P. Middleton, J. R. Hepler, R. Taussig, A. G. Gilman, and S. M. Mumby. Lipid modifications of G proteins: α-subunits are palmitoylated. *Proc. Natl. Acad. Sci. USA* **90:**3675–3679 (1993).
19. Hancock, J. F., A. I. Magee, J. E. Childs, and C. J. Marshall. All ras proteins are polyisoprenylated but only some are palmitoylated. *Cell* **57:** 1167–1177 (1989).
20. Wedegaertner, P. B., D. H. Chu, P. T. Wilson, M. J. Levis, and H. R. Bourne. Palmitoylation is required for signaling functions and membrane attachment of $G_{q\alpha}$ and $G_{s\alpha}$. *J. Biol. Chem.* **268:**25001–25008 (1993).
21. Kennedy, M. E., and L. E. Limbird. Mutations of the *alpha*-2A adrenergic receptor that eliminate detectable palmitoylation do not perturb receptor-G-protein coupling. *J. Biol. Chem.* **268:**8003–8011 (1993).
22. Dowd, B. F., M. Hnatowich, M. G. Caron, R. J. Lefkowitz, and M. Bouvier. Palmitoylation of the β-adrenergic receptor. *J. Biol. Chem.* **264:**7564–7569 (1989).
23. Alland, L., S. M. Peseckis, R. E. Atherton, L. Berthiaume, and M. D. Resh. Dual myristylation and palmitylation of Src family member $p59^{fyn}$ affects subcellular localization. *J. Biol. Chem.* **269:**16701–16705 (1994).
24. Shenoy-Scaria, A. M., D. J. Dietzen, J. Kwong, D. C. Link, and D. M. Lublin. Cysteine 3 of Src family protein tyrosine kinases determines palmitoylation and localization in caveolae. *J. Cell Biol.* **126:**353–363 (1994).
25. Resh, M. D. Myristylation and palmitylation of Src family members: the fats of the matter. *Cell* **76:**411–413 (1994).
26. Kloc, M., B. Reddy, S. Crawford, and L. D. Etkin. A novel 110-kDa maternal CAAX box-containing protein from *Xenopus* is palmitoylated and isoprenylated when expressed in baculovirus. *J. Biol. Chem.* **266:**8206–8212 (1991).
27. Mumby, S. M., C. Kleuss, and A. G. Gilman. Receptor regulation of G-protein palmitoylation. *Proc. Natl. Acad. Sci. USA* **91:**2800–2804 (1994).

Send reprint requests to: Thomas Michel, Cardiovascular Division, Brigham and Women's Hospital, Harvard Medical School, Thorn 1110A, 75 Francis Street, Boston, MA 02115.