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Biochemistry

Mutagenesis of palmitoylation sites in endothelial nitric oxide synthase identifies a novel motif for dual acylation and subcellular targeting

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ABSTRACT **The endothelial nitric oxide synthase (ecNOS) plays a key role in the transduction of signals from the bloodstream to the underlying smooth muscle. ecNOS undergoes a complex series of covalent modifications, including myristoylation and palmitoylation, which appear to play a role in ecNOS membrane association. Mutagenesis of the myristoylation site, which prevents both myristoylation and palmitoylation, blocks ecNOS targeting to cell membranes. Further, as described for some G-protein α subunits, both membrane association and palmitoylation of ecNOS are dynamically regulated: in response to agonists, the enzyme undergoes partial redistribution to the cell cytosol concomitant with depalmitoylation. To clarify the role of palmitoylation in determining ecNOS subcellular localization, we have constructed palmitoylation-deficient mutants of ecNOS. Serine was substituted for cysteine at two potential palmitoylation sites (Cys-15 and Cys-26) by site-directed mutagenesis. Immunoprecipitation of ecNOS mutants following cDNA transfection and biosynthetic labeling with [^{3}H]palmitate revealed that mutagenesis of either cysteine residue attenuated palmitoylation, whereas replacement of both residues completely eliminated palmitoylation. Analysis of N-terminal deletion mutations of ecNOS demonstrated that the region containing these two cysteine residues is both necessary and sufficient for enzyme palmitoylation. The cysteines thus identified as the palmitoylation sites for ecNOS are separated by an unusual (Gly-Leu)$_5$ sequence and appear to define a sequence motif for dual acylation. We analyzed the subcellular distribution of ecNOS mutants by differential ultracentrifugation and found that mutagenesis of the ecNOS palmitoylation sites markedly reduced membrane association of the enzyme. These results document that ecNOS palmitoylation is an important determinant for the subcellular distribution of ecNOS and identify a new motif for the reversible palmitoylation of signaling proteins.**

Acylation has emerged as an important mechanism for the regulation of protein localization and function (1–4). Two distinct forms of protein acylation have been identified: N-myristoylation and palmitoylation. N-myristoylation is a co-translational modification catalyzed by *N*-myristoyltransferase, which modifies a glycine residue within an extensively characterized consensus sequence (1, 4). After removal of the N-terminal methionine residue, myristic acid is attached via amide formation to the amino group of Gly-2. The amide linkage of N-myristoylation is highly stable and generally irreversible under physiological conditions. In contrast, palmitoylation is a reversible, posttranslational modification in which the fatty acid is covalently attached to protein cysteine residues by thioester formation. The fatty acyl thioester linkage is intrinsically more labile than the amide bond of N-myristoylation and is potentially subject to dynamic regulation. Dual acylation, modification by both N-myristoylation and palmitoylation, has been described, notably for members of two families of signaling proteins: G-protein α subunits and Src-related tyrosine kinases (1–4). We have demonstrated that the endothelial nitric oxide synthase (ecNOS) is also both myristoylated and palmitoylated (5, 6).

ecNOS is one of three known isoforms of NOS and catalyzes the production of nitric oxide (NO) from arginine (7–9). Although the isoforms of NOS share many biochemical features, they differ in regulation, tissue distribution, and physiological function. In the vasculature, ecNOS plays a key role in the transduction of signals from the bloodstream to the underlying smooth muscle (10, 11). The calcium/calmodulin-dependent ecNOS is transiently activated by diverse extracellular stimuli that increase intracellular calcium. Endothelium-derived NO activates soluble guanylate cyclase in neighboring vascular smooth muscle cells to effect vasorelaxation and is an important factor in maintenance of blood pressure homeostasis. In vascular endothelial cells, ecNOS is predominantly membrane associated (12). Mutagenesis of the ecNOS myristoylation site, which was found to block both myristoylation and palmitoylation (6), also prevents membrane association (5, 13). The relative importance of myristoylation and palmitoylation in determining ecNOS subcellular distribution remains uncertain. Importantly, in response to agonists such as bradykinin, a fraction of ecNOS translocates from membrane to cytosol (14) in association with enzyme depalmitoylation (6). Acylation may thus play a role in regulating the subcellular distribution of ecNOS and thereby influence the signaling roles of NO.

While the proteins modified by either myristoylation or palmitoylation are numerous and diverse, only a small number of proteins have been identified that undergo both acylation reactions (1–4). Like ecNOS, many of these dually acylated proteins are involved in signal transduction, and regulation of acylation, in particular reversible palmitoylation, may provide an important mechanism for modulating these signaling pathways. Although no general consensus sequence for palmitoylation has been identified, all of the previously characterized dually acylated proteins contain the N-terminal motif Met-Gly-Cys, in which Gly-2 is the site of myristoylation and Cys-3 a site of palmitoylation (1–4). ecNOS, however, lacks this N-terminal consensus sequence (15). We now report the identification of another sequence motif for dual acylation. ecNOS is palmitoylated at two cysteine residues, Cys-15 and Cys-26, which flank an unusual Gly-Leu repeat. Mutagenesis of these cysteines not only blocks palmitoylation of ecNOS but also alters its subcellular distribution, confirming a role for palmitoylation in directing the localization of ecNOS.

METHODS

ecNOS Plasmid Constructs. The plasmid constructs encoding the hemagglutinin (HA) epitope-tagged wild-type or my-

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Abbreviations: NOS, nitric oxide synthase; ecNOS, endothelial isoform of NOS; HA, hemagglutinin.

ristoylation-deficient ecNOS (with Ala substituted for Gly-2) in the expression vector pBK-CMV (Stratagene) have been described (6). For the analysis of ecNOS palmitoylation, site-specific as well as deletion mutants of the ecNOS cDNA were prepared. To substitute Ser for Cys-26 in ecNOS, we used the Muta-Gene Kit (Bio-Rad), according to the manufacturer's protocol, with the mutagenic oligonucleotide 5′-CCTC-GGGCTAAGCGGCAAGCAG-3′ to create pC26S. A Ser codon was substituted for the Cys-15 codon in both the wild-type ecNOS cDNA and in pC26S (to create a double mutant) by PCR under conditions previously described (5). The mutation was introduced by the upstream primer 5′-GC-CCCCCAGCGGCCTGG-3′. The PCR products were digested with *Bss*HII and cloned into the epitope-tagged ecNOS cDNA between the *Ava* I site at bp 50 (treated with DNA polymerase I Klenow fragment) and the *Bss*HII site at bp 279, thus creating pC15S and pC15,26S. The complete nucleotide sequence of the regions synthesized *in vitro* with either the Muta-Gene Kit or PCR were determined to confirm the presence of the desired mutation(s) and the absence of sequence errors.

To construct epitope-tagged deletion mutants of ecNOS, the previously described sequence for the HA epitope tag, followed by a stop codon (6), was subcloned into pBK-CMV between the *Nhe* I and *Kpn* I sites. To create the truncation mutant of ecNOS encoding the N-terminal 528 aa of the protein, pkNT, the 5′ end of the ecNOS cDNA, extending to the *Bsa* I site at bp 1598 and was blunted with the DNA polymerase I Klenow fragment and subcloned upstream from the epitope tag in pBK-CMV. The truncation mutant encoding the C-terminal 694 aa of ecNOS, pkCT, was created by removing the cDNA sequence of the epitope-tagged ecNOS upstream from the *Bgl* II site at bp 1536. The next methionine codon (Met-512) lies within a Kozak consensus sequence favorable for translation initiation. A construct encoding a deletion mutant lacking aa 13–506 (pΔ12–507) was created by removing the sequence in the ecNOS cDNA between the *Ava* I site at bp 50 and the *Bgl* II site at bp 1536, which were joined by blunt-end ligation following treatment with Klenow fragment. A construct encoding a deletion mutant lacking aa 86–506 (pΔ85–507) was created by removing the sequence in the ecNOS cDNA between the *Dra* III site at bp 273 and the *Bgl* II site at bp 1536, which were joined by blunt-end ligation following treatment with T4 DNA polymerase.

Cell Culture and Transfection. COS-7 cells were maintained in culture and transiently transfected by the DEAE-dextran method (5). Cultures were biosynthetically labeled for 2 hr with [^{3}H]palmitate (1 mCi/ml; NEN/DuPont; 1 mCi = 37 MBq) or for 16 hr with [^{3}H]myristate (0.2 mCi/ml, Amersham) in Dulbecco's modified Eagle's medium plus 5% dialyzed fetal bovine serum (GIBCO/BRL), or with [^{35}S]methionine/cysteine (Tran^{35}S-label; ICN), as described (5, 6). After harvesting, cells were sonicated and in some experiments cell lysates were fractionated by ultracentrifugation (5).

Immunoprecipitation and Immunoblotting. Proteins were immunoprecipitated from soluble and particulate fractions or from whole cell lysates with an anti-ecNOS antiserum (5) or the 12CA5 monoclonal antibody (Babco, Emeryville, CA) to the HA epitope (6). Immunoprecipitated proteins were analyzed by SDS/PAGE. Radiolabeled proteins were detected by fluorography, with exposure times of 7–9 days for [^{3}H]palmitate-labeled proteins and 2–4 days for [^{3}H]myristate-labeled proteins. For immunoblotting, proteins separated by SDS/PAGE were transferred electrophoretically to nitrocellulose membranes (Millipore) in 25 mM Capso, pH 10/20% methanol. Anti-ecNOS antiserum was used at a dilution of 1:250, and the 12CA5 antibody to the HA epitope tag, at 1:1000. The nitrocellulose membranes were incubated with the primary antibodies for 60–90 min. The secondary antibodies were peroxidase-conjugated goat anti-rabbit or goat anti-mouse IgG (Pierce) for anti-ecNOS antiserum or the 12CA5 antibody, respectively. The nitrocellulose membranes were incubated with the secondary antibodies, diluted 1:10,000, for 1 hr. Immunoreactive proteins were visualized with the NEN/DuPont Renaissance chemiluminescence reagents.

RESULTS

Mutagenesis of Cys-15 and Cys-26 Blocks ecNOS Palmitoylation. Although no general consensus sequence for protein palmitoylation has been identified, palmitoylation sites are typically located near other points of membrane attachment, near transmembrane segments in the case of integral membrane proteins, or near prenylation or myristoylation sites in proteins containing these modifications (1–3). This observation suggested that the ecNOS palmitoylation site(s) might be found near the N terminus of the protein, the site of myristoylation. Only two cysteine residues (the obligate amino acid for palmitoyl thioester formation) are present within the N-terminal 86 aa of ecNOS: Cys-15 and Cys-26 (15). We therefore replaced each cysteine with serine, individually and in combination, by site-directed mutagenesis (Fig. 1). These mutants were expressed in COS-7 cells by transient transfection and then biosynthetically labeled with [^{3}H]palmitate. Substitution of serine for either Cys-15 or Cys-26 reduced labeling with [^{3}H]palmitate, whereas mutagenesis of both residues completely abolished such labeling (Fig. 2). To confirm that the inhibition of labeling observed in COS-7 cells was not cell-type specific, the ecNOS cysteine mutants were also transiently expressed in bovine aortic endothelial cells, biosynthetically labeled with [^{3}H]palmitate, and selectively immunoprecipitated with the antibody to the HA epitope tag (6). The effects of these cysteine mutations on [^{3}H]palmitate labeling of ecNOS in endothelial cells (data not shown) were identical to those observed in COS-7 cells. Immunoblot analysis of duplicate samples of the immunoprecipitates confirmed that expression of these mutants was at least commensurate with that of the wild-type enzyme (Fig. 2), and NOS catalytic activity (measured by the citrulline assay; ref. 15) in lysates of these cells was the same for the cysteine mutants as for the wild-type enzyme (data not shown). Densitometric analysis of three separate labeling experiments revealed that mutagenesis

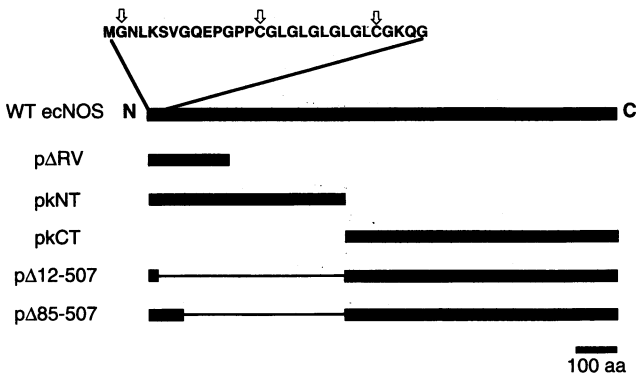

FIG. 1. ecNOS deletion mutants used in the analysis of ecNOS palmitoylation sites. The N and C termini of wild-type (WT) ecNOS are labeled. Shown above the WT ecNOS are the N-terminal 30 aa. Gly-2, the myristoylation site, and Cys-15 and Cys-26, the putative palmitoylation sites, are indicated with arrows. Below WT ecNOS, the deletion mutants are aligned with the corresponding regions of the full-length enzyme. pΔRV encodes the N-terminal sequence of ecNOS, extending to an *Eco*RV site (introduced at bp 498) and was used as a template for site-directed mutagenesis, as described in the text. pkNT encodes the N-terminal domain of ecNOS (aa 1–528), and pkCT the C-terminal domain (aa 512–1205). pΔ12-507 encodes the first 12 aa of ecNOS joined to the C-terminal domain, and pΔ85-507 the first 85 aa of ecNOS joined to the C-terminal domain.

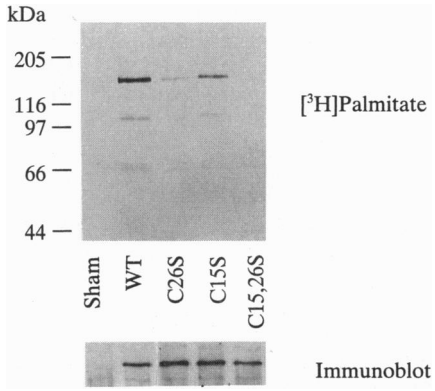

FIG. 2. Palmitoylation of ecNOS cysteine mutants. COS-7 cells were transfected with plasmid vector alone (Sham) or with HA epitope-tagged cDNAs encoding wild-type ecNOS (WT), singly mutated ecNOS (C26S or C15S), or the doubly mutated ecNOS (C15,26S). The transfected cells were biosynthetically labeled with [^{3}H]palmitate, and proteins immunoprecipitated from lysates of these cells with anti-ecNOS antiserum were analyzed by SDS/PAGE. (*Upper*) Fluorogram of ^{3}H-labeled proteins; molecular size markers are noted. Minor protein bands at lower molecular mass may represent proteolytic fragments of ecNOS and/or crossreaction of the ecNOS antibody with nonspecific labeled proteins. (*Lower*) Immunoblot analysis of ecNOS expression. Duplicate samples of the immunoprecipitates were resolved by SDS/PAGE, transferred to nitrocellulose, and probed with anti-ecNOS antiserum. Immunoreactive proteins were detected by chemiluminescence.

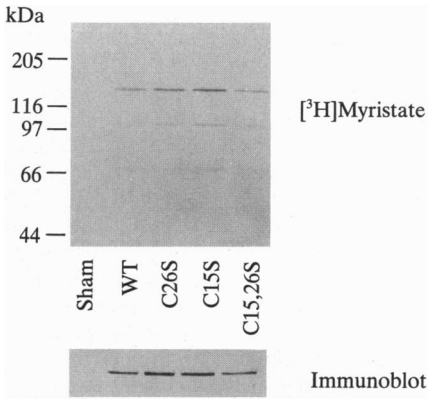

FIG. 3. Myristoylation of ecNOS cysteine mutants. COS-7 cells were transfected with plasmid vector alone (Sham) or with HA epitope-tagged cDNAs encoding wild-type ecNOS (WT), singly mutated ecNOS (C26S or C15S), or the doubly mutated ecNOS (C15,26S). The transfected cells were biosynthetically labeled with [^{3}H]myristate, and proteins immunoprecipitated from lysates of these cells with anti-ecNOS antiserum were analyzed by SDS/PAGE. (*Upper*) Fluorogram of ^{3}H-labeled proteins; molecular size markers are noted. (*Lower*) Immunoblot analysis of ecNOS expression. Duplicate samples of the immunoprecipitates were resolved by SDS/PAGE, transferred to nitrocellulose, and probed with anti-ecNOS antiserum. Immunoreactive proteins were detected by chemiluminescence.

of Cys-15 reduced [^{3}H]palmitate labeling by ≈50%, and mutagenesis of Cys-26 reduced labeling by ≈85%, suggesting an interaction between the putative palmitoylation sites. The residual labeling of the cysteine mutants was sensitive to hydroxylamine at neutral pH (data not shown), indicating linkage via acyl thioester rather than amide formation, as for [^{3}H]palmitate labeling of the wild-type ecNOS (6, 16).

Myristoylation of ecNOS Cysteine Mutants. We previously observed that the myristoylation-deficient mutant of ecNOS also failed to undergo palmitoylation (6). Mutagenesis of Cys-15 and Cys-26, which lie near the N terminus and thus the myristoylation site, might inhibit ecNOS myristoylation and only in consequence disrupt palmitoylation. To investigate this possibility, COS-7 cells transiently transfected with wild-type or cysteine mutants of ecNOS were biosynthetically labeled with [^{3}H]myristate. The palmitoylation mutants of ecNOS clearly underwent myristoylation (Fig. 3). Small variations in the intensity of [^{3}H]myristate labeling were commensurate with variations in the expression of these proteins (Fig. 3), as determined by immunoblotting of duplicate samples. Thus, mutagenesis of Cys-15 and Cys-26 does not inhibit palmitoylation by blocking myristoylation.

Acylation of ecNOS Deletion Mutants. Although the selective inhibition of [^{3}H]palmitate labeling by mutagenesis of Cys-15 and Cys-26 suggested that these residues were the sites of ecNOS palmitoylation, it remained possible that the mutations blocked palmitate labeling by an indirect mechanism. To confirm that Cys-15 and Cys-26 were the direct sites of palmitoylation, we analyzed the pattern of [^{3}H]palmitate labeling for a series of ecNOS deletion mutants (depicted in Fig. 1). First, ecNOS was divided into two truncation mutants: one encoding the N-terminal domain (pkNT, aa 1–528) containing 13 cysteine residues, including Cys-15 and Cys-26, and the other a C-terminal domain (pkCT, aa 512–1205) containing the remaining 17 cysteines of ecNOS. When expressed in transiently transfected COS-7 cells, only the N-terminal mutant (pkNT), like the full-length protein, was palmitoylated (Fig. 4). No [^{3}H]palmitate labeling of the C-terminal mutant (pkCT) was detected despite its robust expression (Fig. 4). These results indicate that ecNOS is palmitoylated within the N-terminal domain, consistent with our identification of Cys-15 and Cys-26 as the palmitoylation sites.

Further experiments were designed to demonstrate that Cys-15 and Cys-26 were not only necessary but sufficient for protein palmitoylation. Since we had observed that the region encoded by pkCT was not palmitoylated, restoration of palmitate labeling by addition of sequences from the N terminus of ecNOS could identify the region of the N-terminal domain containing the palmitoylation sites. To perform such experiments, we prepared additional deletion mutants of ecNOS (shown schematically in Fig. 1) explicitly to test for enzyme

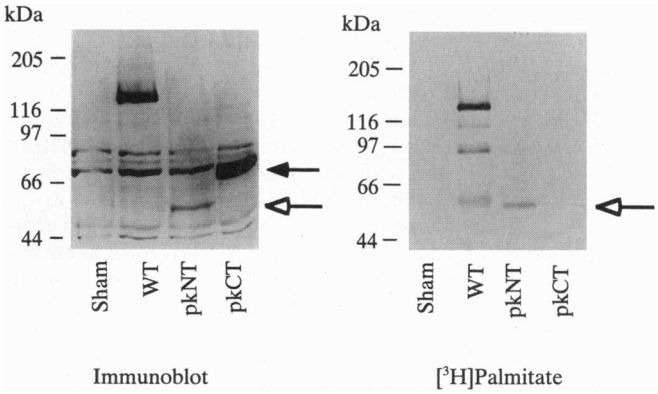

FIG. 4. Expression and palmitoylation of ecNOS truncation mutants. COS-7 cells were transfected with plasmid vector alone (Sham) or with HA epitope-tagged cDNAs for wild-type ecNOS (WT) or truncation mutants encoding only the N-terminal (pkNT) or C-terminal (pkCT) domains of ecNOS. The transfected cells were biosynthetically labeled with [^{3}H]palmitate, harvested, and lysed. (*Left*) Immunoblot analysis of these lysates with the 12CA5 antibody to the epitope tag. After SDS/PAGE and transfer to nitrocellulose, immunoreactive proteins were detected by chemiluminescence. Arrows indicate the predicted migration of full-length ecNOS protein and of the two truncation mutants. Additional bands at 80–90 kDa represent nonspecific proteins crossreacting with the 12CA5 antibody and are present in all lanes. (*Right*) Fluorogram of proteins immunoprecipitated from these cell lysates with the 12CA5 antibody and analyzed by SDS/PAGE. The fluorogram was exposed at −70°C with two intensifying screens to detect labeled proteins.

palmitoylation: Δ12–507, containing the N-terminal 12 aa of ecNOS and thus the myristoylation site but neither putative palmitoylation site, and Δ85–507, containing the N-terminal 85 aa of ecNOS including the myristoylation site and both putative palmitoylation sites, but no other cysteine residues. When expressed in COS-7 cells, both of these mutants, like the wild-type enzyme, were myristoylated, but only Δ85–506 was palmitoylated (Fig. 5). Thus, the addition of Cys-15 and Cys-26 was sufficient to confer palmitoylation on the nonpalmitoylated C-terminal domain. Taken together, the results of site-directed mutagenesis and the analysis of ecNOS deletion mutants demonstrate that Cys-15 and Cys-26 are necessary and sufficient for ecNOS palmitoylation and strongly suggest that these residues are the direct sites of palmitate incorporation.

Subcellular Distribution of ecNOS Acylation Mutants. We have previously shown that, in contrast to the predominantly membrane-associated wild-type enzyme, the myristoylation-site mutant of ecNOS, which is neither myristoylated nor palmitoylated (6), is restricted to the cell cytosol (5, 13). To investigate the relative contributions of myristoylation and palmitoylation to the subcellular targeting of ecNOS, we examined the distribution of the wild-type ecNOS (both myristoylated and palmitoylated), the C15,26S palmitoylation-site mutant (only myristoylated), and the G2A myristoylation-site mutant (neither myristoylated nor palmitoylated). Whereas the G2A mutant, lacking both modifications, was exclusively cytosolic, the palmitoylation-deficient mutant did exhibit some membrane association (Fig. 6). In the absence of palmitoylation, however, ecNOS membrane association was markedly attenuated: densitometric analyses of three independent experiments revealed that loss of palmitoylation alone decreased the percentage of membrane-bound ecNOS from ≈80% to ≈40%, while the further loss of myristoylation eliminated membrane association (to <5%). These results establish roles for both myristoylation and palmitoylation in determining the subcellular localization of ecNOS.

DISCUSSION

These studies identify the sites of ecNOS palmitoylation and demonstrate that this modification is an important determinant of the enzyme's subcellular distribution. Localization of ecNOS at cell membranes may be important for coupling to extracellular signals and may also influence delivery of NO to neighboring cells (7, 8, 10). We had previously observed that mutagenesis of the ecNOS myristoylation site, which blocks both myristoylation and palmitoylation, prevented ecNOS membrane association (5, 6). Thus, acylation of ecNOS appeared to be necessary for membrane binding, but the specific roles of myristoylation and palmitoylation could not be distinguished. In the present studies, comparison of the subcellular distribution of wild-type ecNOS (both myristoylated and palmitoylated) and the palmitoylation-deficient mutant (only myristoylated) revealed a marked reduction in membrane binding in the absence of palmitoylation. Nevertheless, myristoylation alone appeared to confer a limited degree of membrane association on the palmitoylation-deficient mutant (Fig. 6). The myristoyl group might anchor ecNOS to cell membranes via hydrophobic interactions with membrane lipids. However, thermodynamic studies have demonstrated that the free energy of membrane binding for myristoylated peptides or proteins is low, and the interaction is likely to be readily reversible (17). Palmitoylation of ecNOS, by providing additional hydrophobic interfaces, may serve to stabilize the weak membrane association conferred by myristoylation.

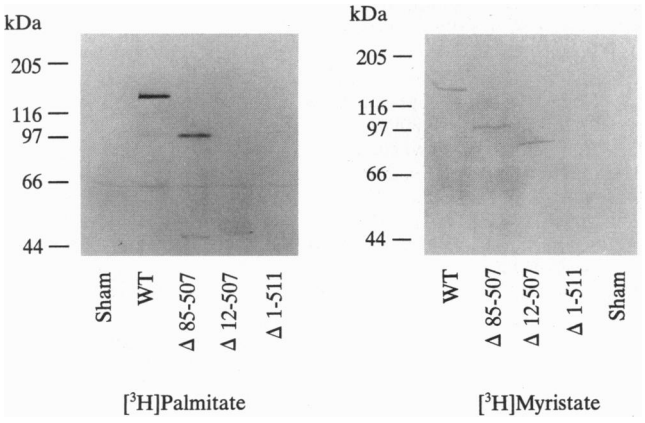

FIG. 5. Acylation of ecNOS deletion mutants. COS-7 cells were transfected with plasmid vector alone (Sham), with the cDNA for wild-type ecNOS (WT), or with constructs encoding mutants of ecNOS (see Fig. 1) lacking amino acids 13–506 (Δ12–507), 86–506 (Δ85–507), or 1–510 (Δ1–511). The transfected cells were biosynthetically labeled with [3H]palmitate (*Left*) or [3H]myristate (*Right*), harvested, and analyzed by immunoprecipitation with the 12CA5 antibody to the epitope tag, followed by SDS/PAGE and fluorography. Treatment of duplicate samples with hydroxylamine at neutral pH removed all of the label from the [3H]palmitate-labeled samples but had no effect on the [3H]myristate labeling. Immunoblots (not shown) of duplicate samples documented the presence of similar levels of protein for the wild-type and mutant ecNOS in these samples.

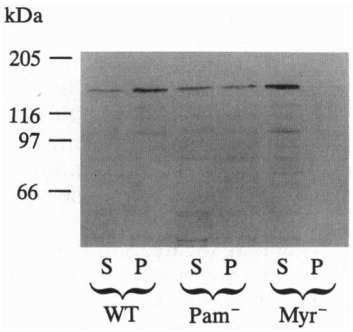

FIG. 6. Subcellular distribution of ecNOS myristoylation- and palmitoylation-deficient mutants. COS-7 cells were transfected with cDNAs encoding wild-type ecNOS (WT), the fully palmitoylation-deficient C15,26S mutant (Pam⁻), or the myristoylation-deficient G2A mutant (Myr⁻). The transfected cells were biosynthetically labeled with [35S]methionine, harvested, lysed, and resolved into soluble (S) and particulate (P) subcellular fractions by ultracentrifugation. Proteins were then immunoprecipitated from these fractions with anti-ecNOS antiserum and analyzed by SDS/PAGE and fluorography. This fluorogram was exposed to x-ray film for 16 hr at −70°C with two intensifying screens. Similar results were obtained in three separate experiments.

Palmitoylation has been suggested to play a similar role in the subcellular targeting of other acylated signaling proteins (1–4): selective inhibition of palmitoylation reduces membrane association for dually acylated proteins, such as the Src-related tyrosine kinase $p59^{fyn}$ (18, 19) and the G-protein subunits α_o and α_i (20, 21). It remains uncertain, though, whether palmitoylation alone can support the membrane association of such proteins. For some proteins, myristoylation may be a prerequisite for palmitoylation, perhaps reflecting its role in membrane targeting (1–3, 22). However, there exist many palmitoylated, but nonmyristoylated, proteins that are membrane associated, including the G-protein subunits α_s and α_q (1–4). For these proteins, other mechanisms may contribute to membrane binding, including additional hydrophobic or electrostatic interactions with membrane phospholipids or associations with other membrane-bound proteins (1–3, 22). Recent work by Venema *et al.* (23) has raised the possibility that the calmodulin-binding domain of ecNOS may play a role in membrane binding by providing electrostatic interactions with negatively charged phospholipids. A similar role has been proposed for the calmodulin-binding domain of the MARCKS

protein, another myristoylated protein exhibiting regulated reversible binding to cell membranes (24). However, for ecNOS, acylation appears to be the primary determinant of ecNOS targeting to the membrane: the cytosolic localization of the myristoylation site mutant of ecNOS (which undergoes neither myristoylation nor palmitoylation) indicates that the enzyme's calmodulin-binding domain, in the absence of acylation, does not permit ecNOS membrane association in cells (5, 23).

In addition to regulating the distribution of ecNOS between membrane and cytosolic compartments, palmitoylation might provide the signal for targeting to a particular organelle. For example, palmitoylation of some Src-related tyrosine kinases may be involved in determining their localization to caveolae (19) and could play a similar role for ecNOS.

We have previously observed that palmitoylation of ecNOS appears to be dynamically regulated: in response to agonists, such as bradykinin, ecNOS depalmitoylation is enhanced (6). The same agonists appear to stimulate ecNOS translocation from membrane to cytosol (14) and might reflect the loss of palmitate and its stabilization of ecNOS membrane binding. Interestingly, the subcellular distribution we now observe for the palmitoylation-deficient ecNOS (Fig. 6) closely resembles that of agonist-treated ecNOS in endothelial cells (14), consistent with the hypothesis that depalmitoylation provides the mechanism for agonist-stimulated release of ecNOS from cell membranes.

The palmitoylated cysteines in ecNOS, residues 15 and 26, flank an unusual 10-aa sequence of alternating glycine and leucine residues. A search of protein sequence databases failed to identify other proteins containing this sequence motif. All other known dually acylated proteins contain the N-terminal consensus sequence Met-Gly-Cys, a sequence not present in ecNOS (1–3, 15). In these proteins, palmitoylation occurs on cysteines adjacent to, or within a few residues of, the myristoylated glycine. In contrast, the modified sites in ecNOS are separated by 10–15 aa and define a new sequence motif for protein acylation; further studies are required to delineate the roles of adjacent amino acid sequences in the control of ecNOS palmitoylation. The spatial distribution of acylation sites relative to the sites of membrane attachment may be critical for recognition of specific cysteines by protein palmitoyltransferase(s) (25). Differences in the spacing or composition of acylation sites might then imply the involvement of distinct palmitoyltransferases. However, analyses limited to the primary structure of palmitoylation sites may fail to identify similarities in their higher-order structure.

Little is known about the substrate specificity of protein palmitoyltransferases and palmitoyl thioesterases. A single protein palmitoyl thioesterase has been cloned and characterized (26, 27), but its relationship to ecNOS palmitoylation is not known. Greater insights into the dynamic modulation of ecNOS signaling are likely to emerge from studies seeking to characterize the specific proteins regulating ecNOS palmitoylation. Palmitoylation has been identified in functionally diverse and structurally dissimilar signaling proteins yet might still provide a mechanism for integrated modulation of cellular signaling functions. Palmitoylation of ecNOS and other signaling proteins by a common palmitoyltransferase could provide a mechanism for coordinate regulation of diverse signaling pathways.

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