A Chimeric Transmembrane Domain Directs Endothelial Nitric-oxide Synthase Palmitoylation and Targeting to Plasmalemmal Caveolae*

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The endothelial nitric-oxide synthase (eNOS), a key signaling protein, undergoes a series of covalent modifications, including co-translational N-myristoylation at Gly2, as well as post-translational thiopalmitylation at Cys15 and Cys26. Myristoylation of eNOS is required for the subsequent palmitylation of the enzyme, and both acylations are required for the efficient subcellular targeting of eNOS to plasmalemmal caveolae. We constructed chimeric cDNAs encoding proteins comprised of various acylation-deficient eNOS mutants fused at their N termini to the hydrophobic transmembrane domain of the glycoprotein CD8 and characterized these constructs in transient transfection experiments in COS-7 cells. One construct (termed CD8-myr"eNOS) encodes a fusion protein comprised of the eNOS myristoylation-deficient mutant coupled to the CD8 transmembrane domain. In biosynthetic labeling experiments using [³H]palmitic acid, we found that the CD8-myr"eNOS chimera underwent palmitylation. Subcellular fractionation showed that the CD8-myr"eNOS chimera is targeted to caveolae. We also constructed and characterized a cDNA encoding the CD8 transmembrane domain fused to the palmitylation-deficient mutant eNOS (in which Cys15 and Cys26 are changed to serine). This chimera (termed CD8-myr"palm eNOS) did not undergo palmitylation, indicating that the palmitylation seen with the CD8-myr"eNOS fusion protein occurs on the same residues as in the wild-type enzyme. Importantly, the CD8-myr"palm eNOS fusion protein remained efficiently targeted to caveolae, in contrast to the palm"eNOS mutant lacking the CD8 transmembrane domain, which has no native caveolar localization. A construct encoding the CD8 transmembrane domain alone was insufficient for selective targeting to caveolae. These results indicate that membrane targeting per se, but not necessarily myristoylation, is sufficient for eNOS palmitylation and localization to plasmalemmal caveolae, and suggest further that sequences within eNOS itself, in addition to its palmitylation sites, facilitate the selective localization of the enzyme within caveolae.

Acylation modulates the subcellular targeting of many structurally distinct signaling proteins to microdomains within the plasma membrane termed caveolae (1–4). N-Myristoylation and thiopalmitylation represent two distinct forms of protein acylation (1, 3). Myristoylation is a co-translational modification catalyzed by N-myristoyltransferase, an enzyme that modifies a specific glycine residue (Gly2) within a consensus sequence at the N terminus of the protein via the formation of an irreversible fatty acyl amide bond involving the Gly2 amino group (1, 3). Myristoylation alone is usually not sufficient to promote efficient targeting of peripheral membrane proteins to the plasmalemma, and many myristoylated proteins are further stabilized in their membrane association by other intermolecular interactions and/or covalent modifications, including palmitylation.

Protein palmitylation involves the post-translational formation of a fatty acyl thioether between palmitoyl-CoA and specific cysteine residue(s) in the modified protein (2). In contrast to myristoylation, there is no clear consensus sequence for protein palmitylation (1). Rather, the sites of palmitylation within a given membrane-targeted protein characteristically occur at cysteine residues located in proximity to sites of the protein’s membrane attachment, and enzymes that catalyze protein thiopalmitylation have not been definitively identified. Indeed, there is evidence that protein palmitylation can occur without the involvement of a separate protein catalyst (2), although a protein palmitylthioesterase that depalmitylates the G protein Ga (5) as well as eNOS (6) has recently been identified. In contrast to myristoylation, which is characteristically an irreversible covalent modification, the protein palmitoyl thioester bond is labile; the palmitylation of several signaling proteins can be dynamically regulated by agonists (7). Some examples of palmitylated signaling proteins include G protein-coupled receptors, α subunits of heterotrimeric G proteins, diverse nonreceptor tyrosine kinases, and the endothelial nitric-oxide synthase (eNOS) (7).

The eNOS plays a critical role in controlling vascular tone, platelet aggregation, and cardiac myocyte function (8). In vascular endothelial cells and cardiac myocytes, eNOS is targeted to plasmalemmal caveolae (for review see Ref. 9), where the enzyme interacts with the scaffolding protein caveolin. The eNOS protein contains no hydrophobic transmembrane domain, and the enzyme is targeted to plasmalemmal caveolae by virtue of dual acylation: by N-myristoylation at Gly2 and by

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1 The abbreviations used are: eNOS, endothelial nitric-oxide synthase; myr, myristoylation-deficient; palm, palmitylation-deficient; PAGE, polyacrylamide gel electrophoresis; CEM, caveolin enriched membranes; NCM, noncaveolar membranes; CHAPS, 3-[3-holamido(propyl)dimethylammonio]-1-propanesulfonic acid; MES, 2-(N-morpholino)ethanesulfonic acid.
thiopalmitylation at Cys^{15} and Cys^{26} (10). In vitro mutagenesis of the myristoylation site of eNOS, in which Gly^{2} is changed to alanine, yields a myristoylation-deficient eNOS (myr^{-} eNOS) mutant that is neither myristoylated nor palmitylated and is recovered in the soluble subcellular fraction when expressed in cells (10, 11). Because the myr^{-} mutant of eNOS remains in the cytosol and does not undergo palmitylation (despite the presence of intact cysteine residues at its sites of palmitylation), it is difficult to discern the relative contribution of myristoylation and palmitylation in targeting the enzyme to caveolae. We therefore constructed a series of fusion proteins between various eNOS acylation mutants and a prototypical transmembrane domain (derived from the T cell surface glycoprotein CD8) and characterized the post-translational modifications and targeting of these chimeric constructs. The presence of a transmembrane domain in this chimeric protein provides an alternative mechanism whereby eNOS may be targeted to caveolae and/or undergo palmitylation. Our results indicate the presence of the CD8 transmembrane domain is sufficient to promote eNOS palmitoylation and targeting to caveolae. Furthermore, we show that palmitylation of eNOS is not required for the selective targeting of a CD8:eNOS fusion protein to plasmalemmal caveolae.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction—**cDNA constructs encoding wild-type eNOS, myristoylation-deficient (myr^{-}) eNOS, and palmitylation-deficient (palm^{-}) eNOS have been previously described (10). Fig. 1 shows the various eNOS acylation mutants and CD8:eNOS chimeras used in these studies. The CD8:myr eNOS DNA was previously constructed (12) in an adenovirus transfer vector as a fusion protein between the glycoprotein CD8 transmembrane domain and the myr^{-} eNOS cDNA. This CD8:myr eNOS insert was excised from the adenovirus transfer vector (12) by digestion with EcoRI and ligated into the EcoRI site of the human expression vector pCMV, a derivative of pHCMV (Stratagene) modified as described previously (10). To construct the palmitylation-deficient CD8:myr^{-}palm^{-} eNOS fusion protein, a cassette comprising ~1.5 kilobases of the CD8:myr eNOS cDNA was exchanged for the corresponding region of the palm^{-}eNOS cDNA and excised using the restriction enzymes SfI and BglII, yielding the plasmid CD8:palm^{-}eNOS. The CD8 transmembrane domain encodes a polypeptide with a predicted M_{r} ~ 22,000, and the chimeric constructs of CD8 plus eNOS (M_{r} ~ 135,000) yield fusion proteins of M_{r} ~ 157,000 (Fig. 2).

cDNA encoding the CD8 transmembrane domain region was amplified by polymerase chain reaction from the CD8:myr^{-}eNOS plasmid using forward primer 5'-GATTCATCGGTTCCTGTGGTT-3' and the reverse primer 5'-ATCGATTCATCGGTTCCTGTGGTT-3'. The resulting polymerase chain reaction product, containing with EcoRI and ClaI and subcloned into the mammalian expression vector pBK-CMV (Stratagene). The sequence of this polymerase chain reaction-generated fragment was confirmed by dideoxy nucleotide sequencing, and the construct directed the expression of a protein of the expected size (~22 kDa) that was detected by the CD8 N terminus-specific antibody (Santa Cruz Biotechnology).

Cell Culture and Transient Transfection of COS-7 Cells—COS-7 cells were maintained in culture as described previously (11) and were transfected with 1–5 μg of total plasmid DNA in 100-mm cell culture plates, using Lipofectamine (Life Technologies, Inc.) according to the manufacturer's protocols.

Preparation of Cell Lysates and Subcellular Fractionation—Cells were harvested by scraping in phosphate-buffered saline. After centrifugation, the cell pellet was resuspended in buffer 1 (50 mM Tris-HCl (pH 7.4), 0.1 mM EDTA, 0.1 mM EGTA, 2 mM β-mercaptoethanol plus protease inhibitors; linae bean trypsin inhibitor, soy bean trypsin inhibitor, leupeptin, and antipain, each at final concentration of 2 μg/ml). Cells were sonicated (three 10-s bursts with 10-s intervals, output power at 10% of nominal converter amplitude) using a Branson 450 sonifier (Branson Ultrasonic, Danbury, CT) to yield the cell lysate. Cell debris was removed following a brief 1000 × g centrifugation. When subcellular fractionation was required, cells were sonicated in buffer 1, and the resulting lysates were centrifuged to separate particulate and soluble fractions, in an ultracentrifuge (100,000 × g) for 1 h, at 4 °C. Protein concentrations were determined using the Bradford reagent (Bio-Rad).

**RESULTS AND DISCUSSION**

**Expression of CD8:myr^{-}eNOS and CD8:myr^{-}palm^{-}eNOS Fusion Proteins—**The expression and targeting of the CD8:myr eNOS fusion proteins were evaluated in transient transfection experiments in COS-7 cells using the cDNA constructs shown in Fig. 1. As a preliminary characterization, we performed a subcellular fractionation of lysates of transfected cells using ultracentrifugation at 100,000 × g to resolve the particulate cell pellet and the soluble supernatant fractions (Fig. 2). The wild-type eNOS was recovered principally in the particulate subcellular fraction (Fig. 2), and the myr^{-} mutant eNOS was primarily in the supernatant (data not shown), as we have previously reported (10, 11). The palm^{-} mutant of
eNOS, which still undergoes N-myristoylation (10, 11), showed a subcellular distribution intermediate between the wild-type enzyme (myristoylated and palmitoylated) and the myr mutant, which is entirely acylation-deficient. However, in the CD8 fusion proteins, the presence of the CD8 transmembrane domain targeted the different eNOS mutants to the particulate subcellular fraction irrespective of the status of the acylation sites of the enzyme in the chimeric constructs. The truncated CD8 transmembrane domain was also recovered in the particulate fraction (Fig. 2B). The overall nitric-oxide synthase activity in these lysates (assayed using the [3H]arginine → [3H]citrulline assay, Ref. 20) ranged from 1–3 pmol of [3H]citrulline formed/min/mg of protein in different preparations, and the distribution of nitric-oxide synthase enzyme activity between the soluble and particulate fractions paralleled the distribution of nitric-oxide synthase protein in the different subcellular fractions. These results indicate that the fusion of the CD8 transmembrane domain to eNOS can provide efficient targeting of the chimera to the particulate subcellular fraction; the loss of acylation sites in the eNOS N-terminal is entirely overcome by the addition of an N-terminal transmembrane domain, which yields a membrane-localized chimeric enzyme.

Palmitoylation of Chimeric Fusion Proteins—The myristoylation-deficient mutant eNOS does not undergo palmitoylation, suggesting that myristoylation is required for eNOS palmitoylation (13). Because the CD8 transmembrane domain provides an alternative mechanism whereby the chimeric CD8-myristoylation-deficient eNOS fusion protein targets to the particulate fraction (Fig. 2), we explored whether this chimeric construct might also undergo palmitoylation. cDNA constructs encoding the various CD8-eNOS chimeric proteins were transiently expressed in COS-7 cells. Transfection of the wild-type eNOS served as a positive control, and the palm mutant eNOS served as a negative control. The transfected cells were biosynthetically labeled with [3H]palmitate, and soluble cell lysates were immunoprecipitated with anti-eNOS antiserum (11); proteins were resolved by SDS-PAGE and detected by fluorography. As shown in Fig. 3, the wild-type eNOS is palmitoylated, whereas the palm eNOS mutant enzyme is not; these are the expected results (10). When COS-7 cells were transfected with plasmid cDNAs encoding the CD8-myr eNOS fusion protein, immunoprecipitation of eNOS revealed the incorporation of [3H]palmitate into the chimera, although the chimera is more weakly [3H]labeled than the wild-type enzyme (Fig. 3). Importantly, the biosynthetic labeling of cells transfected with the CD8-myr-palm eNOS construct, in which the palmitoylation sites at Cys15 and Cys26 are mutated to alanine residues, showed no incorporation of the [3H] label. These results strongly indicate palmitoylation of the CD8-myr eNOS occurs on the same residues as those that undergo palmitoylation in the wild-type enzyme and establish that the presence of the CD8 transmembrane domain alone is sufficient to promote eNOS palmitoylation of the myristoylation-deficient enzyme.

To further validate that the incorporation of [3H]palmitate reflects the thiopalmitoylation of eNOS (rather than biosynthetic incorporation of labeled palmitate-derived amino acids), we used hydroxylamine to treat SDS-PAGE gels containing the biosynthetically labeled immunoprecipitated eNOS. As can be seen in Fig. 3B, the [3H]palmitate label was completely removed by hydroxylamine treatment, suggesting that the label-
ing reflects thioester formation from the incorporation of \(^{[3]H}\)palmitic acid.

These results indicate that membrane localization, rather than myristoylation per se, is sufficient to support the palmitoylation of eNOS. Palmitoylated proteins, which are almost invariably membrane-associated (1, 2), undergo targeting to membrane fractions via diverse pathways; prenylated proteins, myristoylated proteins, and intrinsic membrane proteins may all contain cysteine residues that undergo palmitoylation. The present study establishes that a transmembrane domain is able to substitute for N-myristoylation and lead to the palmitoylation of a protein. Despite the proximity of a large chimeric transmembrane domain in place of the myristoylated glycine residue of the wild-type protein, the eNOS-CD8 chimera nevertheless undergoes palmitoylation, thereby providing further evidence that the proximity of a given cysteine residue to the site of membrane attachment is the principal determinant of its propensity to undergo thioesterification.

**Caveolar Localization**—The localization of the different chimeric CD8-eNOS constructs to the particulate subcellular fraction obviously does not discriminate whether these proteins are specifically targeted to plasmalemmal caveolae. Our next series of experiments analyzed the subcellular distribution of the various transfected constructs in greater detail. We applied an isopycnic centrifugation method (14–16) that resolves caveolae in carbonate-extracted cell lysates separated in discontinuous sucrose gradients to discriminate CEM versus NCM. Using this methodology, ~80% of the caveolin-1 protein, but only 3% of the total cellular protein, is recovered in the CEM (Fig. 4), suggesting that this method reliably resolves caveolae-enriched from noncaveolar membranes. As shown in Fig. 4, the wild-type eNOS, as well as the CD8-myrm eNOS and CD8-myrm-palm eNOS fusion proteins, are recovered principally in the CEM fraction. Indeed, the CD8-myrm eNOS fusion protein was even more efficiently targeted to CEM than was the wild-type enzyme (55 ± 7% targeted to CEM for the wild-type eNOS versus 78 ± 8% for the CD8-myrm eNOS chimera, \(p < 0.05\) by ANOVA [analysis of variance], \(n = 3\)). By contrast, the palm eNOS was recovered principally in the noncaveolar fraction, although some enzyme was always recovered in the CEM fraction (17 ± 8%, \(n = 3\)). The fusion protein between CD8 and the palmitoylation-deficient mutant eNOS was targeted to the caveolin-enriched membranes as efficiently as the palmitoylation-competent chimeric CD8 eNOS (65 ± 7% for the CD8-myrm-palm and 78 ± 8% for the CD8-myrm-NOS constructs; \(p\) value not significant, \(n = 4\)). Transfection experiments using the construct encoding only the CD8 transmembrane domain (i.e. not as a fusion protein with eNOS) showed that this transmembrane domain was recovered almost exclusively in the NCM fraction, indicating that specific sequences within the CD8 moiety were not responsible for directing the targeting of eNOS to the CEM fraction. Taken together, these results suggest that the CD8 transmembrane domain alone is insufficient for targeting to caveolae, but that the CD8-eNOS fusion proteins are even more efficiently targeted to caveolae than the wild-type enzyme. Therefore, the acylation of eNOS, once the enzyme is targeted to the membrane by virtue of the CD8 transmembrane domain, appears to play a relatively minor role in further enhancing targeting to caveolae. It must be noted that the targeting of wild-type eNOS to caveolae is importantly influenced by the status of the palmitoylation of the enzyme, which itself is dynamically regulated in the cell. However, caveolar localization of eNOS can occur in the absence of palmitoylation, so long as the protein finds its way to the plasmalemna by some other means.

**Association of Chimeric Fusion Proteins with Caveolin**—We have previously used co-immunoprecipitation techniques to investigate the associations between eNOS acylation mutants and caveolin (17–19, 21, 22). As shown in Fig. 5, there were significant differences between the various eNOS constructs in their interaction with caveolin-1, as assessed by co-immunoprecipitation. These co-immunoprecipitation experiments were controlled for equivalent protein expression by immunoblot analyses, and the specificity of the interaction was validated by the absence of co-immunoprecipitation in the sham-transfected cells (Fig. 5). The chimeras CD8-myrm-eNOS and CD8-myrm-palm eNOS, as well as the wild-type enzyme all are efficiently co-immunoprecipitated with caveolin, whereas there was considerably less efficient co-immunoprecipitation of palm eNOS. The current report complements an earlier study in which caveolin overexpression was shown to lead to the association between caveolin and various eNOS acylation mutants (21), suggesting that the proximity of eNOS and caveolin could be sufficient to permit their associating within the cell. Taken together, these results indicate that the ability of nitric-oxide synthase to interact with caveolin may be independent of the state of the enzyme’s state of acylation per se but rather may be determined principally by 1) the relative partitioning of eNOS into the cell membrane and 2) by the presence of sequences within eNOS that are involved in its association with caveolin.
For the wild-type eNOS, membrane partitioning is critically influenced by palmitoylation (10). Depalmitoylation of eNOS may promote its translocation principally by affecting the relative affinity of the enzyme for the cell membrane, thereby changing its proximity to, but not necessarily its affinity for, caveolin.

Implications for Targeting of Signaling Proteins to Caveolae—Palmitoylated proteins are almost invariably found in the particulate subcellular fraction (23, 24), and the preponderance of palmitoylated signaling proteins can be found localized in plasmalemmal caveolae (7). The fact that membrane targeting via a transmembrane domain, rather than palmitoylation, leads to selective targeting to caveolae suggests that this acylation per se is not required for the selective targeting of eNOS to caveolae. On the other hand, sequences within eNOS are evidently required for targeting to caveolae and its interaction with caveolin in that the CD8 transmembrane domain by itself neither selectively localizes in caveolae nor co-immunoprecipitates with caveolin.

If targeting to caveolae does not depend on palmitoylation, then why are so many palmitoylated proteins found in caveolae? Possibly, the fact that many of the signaling proteins targeted to caveolae undergo palmitoylation could represent a mechanism whereby the proteins may leave caveolae when depalmitoylated (as a consequence of their loss of hydrophobic acyl chains). However, if a transmembrane hydrophobic domain is the determinant obligating a protein’s stable association with the plasmalemma, the additional presence of high affinity caveolin-binding domains within the protein could lead to its irreversible targeting to caveolae. A consensus sequence for caveolin binding has been found in a wide variety of signaling proteins (25), including intrinsic as well as peripheral membrane proteins targeted to caveolae; the larger role of this consensus sequence in caveolar targeting has not yet been clearly defined. Indeed, many such intrinsic membrane proteins, including diverse growth factor receptors and G protein-

![Diagram](https://example.com/diagram.png)

**Fig. 4. Distribution of eNOS chimeras in caveolar and non-caveolar membrane fractions.** Shown are the results of subcellular fractionation and immunoblot analyses of COS-7 cells transfected with cDNA encoding wild-type eNOS (wt-eNOS), palm eNOS, CD8-myr eNOS, CD8-myr-palm eNOS, or the CD8 transmembrane domain (CD8-TMD), as noted. Subcellular fractionation of the transfected cells was performed as described in the text; fractions corresponding to CEM and NCM were pooled and analyzed by SDS-PAGE, and immunoblots were probed using eNOS, caveolin, or CD8 antibodies as indicated in the figure. A shows results from representative immunoblot experiments, and B shows pooled results from four independent experiments analyzed by densitometry (statistical significance was determined by ANOVA followed by Scheffe’s F-test. A p value <0.05 was considered as statistically significant).

**Fig. 5. Co-immunoprecipitation of caveolin and eNOS chimeras.** Shown are results of co-immunoprecipitation/immunoblot experiments in COS-7 cells co-transfected with cDNA encoding Myc-epitope-tagged caveolin-1 plus either wild-type eNOS (wt-eNOS), palm eNOS, CD8-myr eNOS, or CD8-myr-palm eNOS proteins, as shown. A shows the results of eNOS and caveolin immunoblots in the cellular lysates. The lower panel in B shows the results of co-immunoprecipitation experiments in which immune complexes are precipitated using the Myc antibody (which recognizes the epitope tag in the caveolin construct) and analyzed using immunoblots probed with eNOS or caveolin antibodies. This figure is representative of three independent experiments.
coupled receptors, have been found to target to caveolae. Some G protein-coupled receptors have been found to be reversibly palmitoylated; the relationship between the reversible palmitoylation of these receptors and their dynamic targeting to caveolae remains less well understood. Clearly there are many pathways a protein may take on the path to or from plasmalemmal caveolae. A broader understanding of the determinants of this process may provide important insights into the regulation of caveolae-associated proteins in normal and pathological states.

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