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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 Gly^2, as well as post-translational thiopalmitoylation at Cys^{15} and Cys^{26}. Myristoylation of eNOS is required for the subsequent palmitoylation 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 [^{3}H]palmitic acid, we found that the CD8-myr$^-$eNOS chimera undergoes palmitoylation. 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 palmitoylation-deficient mutant eNOS (in which Cys^{15} and Cys^{26} are changed to serine). This chimera (termed CD8-myr$^-$·palm$^-$eNOS) did not undergo palmitoylation, indicating that the palmitoylation 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 nominal 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 palmitoylation and localization to plasmalemmal caveolae, and suggest further that sequences within eNOS itself, in addition to its palmitoylation 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 thiopalmitoylation 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 (Gly^2) within a consensus sequence at the N terminus of the protein via the formation of an irreversible fatty acyl amide bond involving the Gly^2 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 palmitoylation.

Protein palmitoylation involves the post-translational formation of a fatty acyl thioester 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 palmitoylation (1). Rather, the sites of palmitoylation 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 thiopalmitoylation have not been definitively identified. Indeed, there is evidence that protein palmitoylation can occur without the involvement of a separate protein catalyst (2), although a protein palmitoylthioesterase that depalmitoylates the G protein Gα_s (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 palmitoylation of several signaling proteins can be dynamically regulated by agonists (7). Some examples of palmitoylated signaling proteins include G protein-coupled receptors, α subunits of heterotrimeric G proteins, diverse nonreceptor tyrosine kinases, and the endothelial nitric-oxide synthase (eNOS)[1] (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 Gly^2 and by

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[1] The abbreviations used are: eNOS, endothelial nitric-oxide synthase; myr$^-$, myristoylation-deficient; palm$^-$, palmitoylation-deficient; PAGE, polyacrylamide gel electrophoresis; CEM, caveolin enriched membranes; NCM, noncaveolar membranes; CHAPS, 3-[(3-holamidopropyl)dimethylammonio]-1-propanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid.

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thiopalmitoylation 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 palmitoylated 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 palmitoylation (despite the presence of intact cysteine residues at its sites of palmitoylation), it is difficult to discern the relative contribution of myristoylation and palmitoylation 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 palmitoylation. Our results indicate the presence of the CD8 transmembrane domain is sufficient to promote eNOS palmitoylation and targeting to caveolae. Furthermore, we show that palmitoylation 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 palmitoylation-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 *Eco*RI and ligated into the *Eco*RI site of the mammalian expression vector pK, a derivative of pBK-CMV (Stratagene) modified as described previously (10). To construct the palmitoylation-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 *Srf*I and *Bgl*II, yielding the plasmid CD8-$palm^-$·myr^-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′-GAATTCGAGCCAAGCAGCGTCCTGGGG-3′ and the reverse primer 5′-ATCGATTCATCGGTTCCTGTGGTT-3′. The resulting polymerase chain reaction product was digested with *Eco*RI and *Cla*I 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: lima 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).

Biosynthetic Labeling—Transfected COS-7 cells were biosynthetically labeled for 2 h with [^{3}H]palmitate (1 mCi/ml; NEN Life Science Products) in Dulbecco's modified Eagle's medium plus 10% dialyzed fetal bovine serum (Life Technologies, Inc.) as described previously (13). Cells were harvested as described above and then sonicated in buffer B (10 mM Tris-HCl, 1 mM EDTA, 0 1% SDS, 1% Triton, 1% sodium deoxycholate, 100 mM NaCl, and protease inhibitors as described above).

Immunoprecipitation and Autoradiography—Immunoprecipitation of eNOS was done in Buffer-B using a polyclonal antiserum against eNOS as described previously (11). Immunoprecipitated proteins were eluted from the protein A-Sepharose using a modified Laemmli sample buffer containing 5 mM dithiothreitol as reducing agent instead of β-mercaptoethanol (to minimize loss of the palmitate thioester). Proteins were separated by SDS-PAGE in 7.5% gels, which were then fixed and soaked in EN^{3}HANCE (DuPont); radiolabeled proteins were detected in the dried gels by fluorography using XAR film (Kodak), with an average exposure time of 7 days.

Preparation of Caveolin-enriched Membrane Fractions (CEM)—CEM and noncaveolar membranes (NCM) were resolved by a detergent-free ultracentrifugation method (14–16). Transfected cells from a 100-mm dish were harvested in 2.2 ml of 0.5 M sodium bicarbonate, pH 11, and homogenization was carried out sequentially with a loose fitting Dounce homogenizer (40 strokes) and a sonicator (3 × 20 s bursts). The extract was brought to 45% sucrose (in MES-buffered saline buffer: 25 mM MES, 150 mM NaCl, pH 6.5) by adding 2 ml of homogenate to 2 ml of 90% sucrose solution and placed at the bottom of a 12-ml ultracentrifuge tube. A discontinuous gradient was formed above the 45% lysate solution by adding 4 ml of 35% and 4 ml of 5% sucrose solutions prepared in MES-buffered saline containing 0.25 M sodium carbonate. Tubes were centrifuged at 39,000 rpm in a SW-41 rotor for 16–18 h at 4 °C. After centrifugation, 15 × 0.8-ml fractions were collected from the top of each gradient. To concentrate the membranes within different regions of the gradient, fractions corresponding to CEM (fractions 4–7) and NCM (fractions 8–15) were pooled and spun at 100,000 × *g* for 1 h at 4 °C. The pelleted membrane proteins were solubilized in the solubilization buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 60 mM β-octylglucoside, 1% Triton X-100) and protease inhibitors mentioned above. Laemmli sample buffer (2×) was added in equal volume to the solubilized membranes, and equal amounts of proteins from CEM and NCM were separated on SDS-PAGE for the detection of eNOS, caveolin, and the CD8 transmembrane domain.

Co-immunoprecipitation—Co-immunoprecipitations of eNOS and caveolin from transfected COS were was performed in CHAPS buffer (containing 20 mM CHAPS, 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 1 mM EGTA, plus protease inhibitors as above) using our previously reported protocols (17–19). Briefly, solubilized cell lysates were incubated with rabbit polyclonal anti-Myc antibody (2 μg/mg protein; Santa Cruz Biotechnology) for 1.5 h. Protein A-Sepharose was then added for 1 h, and immunoprecipitated complexes were washed three times with the CHAPS buffer and eluted by boiling in Laemmli sample buffer. Proteins were separated by SDS-PAGE using 7% gels and were transferred to nitrocellulose membrane by electroblotting, as described previously (18, 19). For immunodetection of eNOS and caveolin, monoclonal and polyclonal antibodies (Transduction Laboratories) directed against eNOS and caveolin, respectively, were used for chemiluminescent detection of proteins as described previously (17). Immunoblotting of Myc-tagged caveolin-1 and CD8-transmembrane domain was performed using monoclonal anti-Myc antibodies and polyclonal anti-CD8 (N terminus-specific; H-160) antibody, respectively, from Santa Cruz Laboratories, according to the manufacturer's protocols.

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

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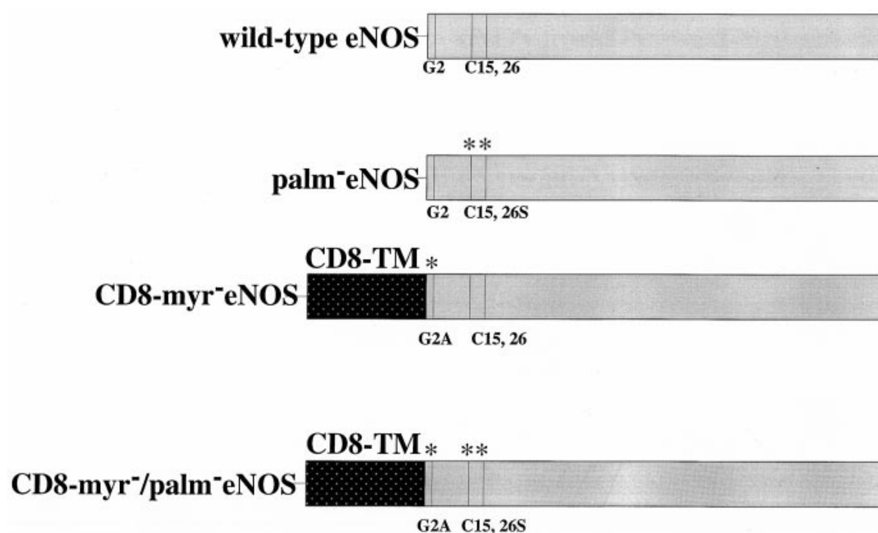

FIG. 1. **eNOS acylation mutants and chimeras.** The full-length wild-type eNOS protein (135 kDa) is shown with its N-terminal myristoylation site at Gly^2, and two palmitoylation sites, at Cys^{15} and Cys^{26}. In the palm−eNOS construct, these two palmitoylation site cysteine residues are mutated to alanine. The CD8·myr−eNOS is a 157-kDa fusion protein in which the CD8 transmembrane domain is fused to the N terminus of myristoylation-deficient eNOS; this construct retains the two palmitoylated cysteine residues of the enzyme. However, in the CD8-myr$^-$ ·palm$^-$eNOS, the cysteine residues in the CD8·myr$^-$eNOS fusion protein are changed to serine to generate an acylation-deficient mutant in which the sites for myristoylation and palmitoylation are inactivated.

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. 2*B*). The overall nitric-oxide synthase activity in these lysates (assayed using the [^{3}H]arginine → [^{3}H]citrulline assay, Ref. 20) ranged from 1–3 pmol of [^{3}H]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 [^{3}H]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 [^{3}H]palmitate into the chimera, although the chimera is more weakly ^{3}H-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 Cys^{15} and Cys^{26} are mutated to alanine residues, showed no incorporation of the ^{3}H 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.

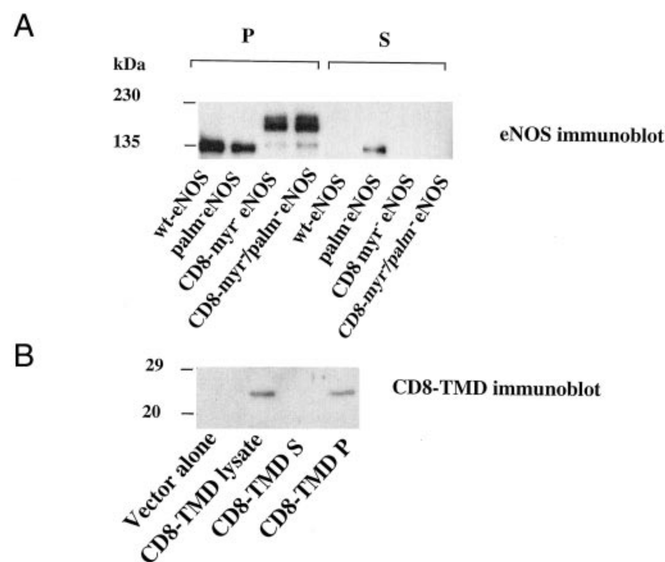

FIG. 2. **Expression and subcellular fractionation of eNOS acylation mutants and chimeras.** Shown is an immunoblot of transfected COS-7 cells probed either with eNOS antibodies (*A*) or CD8 N terminus antibodies (*B*). COS-7 cells were transfected as indicated in the figure with cDNAs encoding wild-type eNOS (*wt-eNOS*), palm$^-$eNOS, CD8-myr$^-$eNOS, or CD8-myr$^-$·palm$^-$eNOS (*A*) or with empty vector or the CD8-transmembrane domain (*CD8-TMD*, *B*). Lysates prepared from the transfected cells were resolved into particulate (*P*) and soluble (*S*) subcellular fractions by ultracentrifugation, as described in the text, separated on SDS-PAGE, and analyzed by immunoblotting. The blot is a representative of three independent experiments.

To further validate that the incorporation of [^{3}H]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. 3*B*, the [^{3}H]palmitate label was completely removed by hydroxylamine treatment, suggesting that the label-

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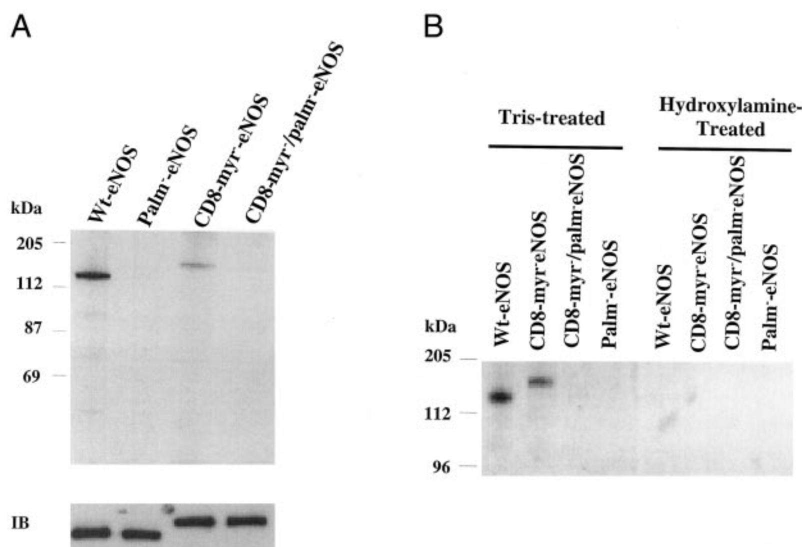

FIG. 3. **Palmitoylation of eNOS acylation mutants and chimeras.** Shown are analyses of eNOS immunoprecipitated from transfected COS-7 cells biosynthetically labeled with [^{3}H]palmitate. COS-7 cells were transfected with cDNAs encoding wild-type eNOS (*wt-eNOS*), palm$^-$eNOS, CD8-myr$^-$eNOS, or CD8-myr$^-$·palm$^-$eNOS, as indicated. The transfected cells were biosynthetically labeled with [^{3}H]palmitate, harvested, immunoprecipitated with anti-eNOS antiserum, resolved by SDS-PAGE, and analyzed by fluorography. *A* shows a fluorogram of eNOS immunoprecipitated from transfected/biosynthetically labeled cells. The panel labeled "*IB*" shows the results of an immunoblot of samples from the same experiment probed with eNOS antibody, validating that eNOS is expressed at an equivalent level for the different constructs. *B* shows a separate experiment in which the gel was treated with either Tris buffer or hydroxylamine, as noted in the figure.

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 proclivity to undergo thiopalmitoylation.

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 caveolin-enriched from noncaveolar membranes. As shown in Fig. 4, the wild-type eNOS, as well as the CD8·myr$^-$eNOS and CD8·myr$^-$·palm$^-$eNOS fusion proteins, are recovered principally in the CEM fraction. Indeed, the CD8·myr$^-$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·myr $^-$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·myr$^-$·palm$^-$ and 78 ± 8% for the CD8·myr$^-$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 plasmalemma 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·myr$^-$eNOS and CD8·myr$^-$·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.

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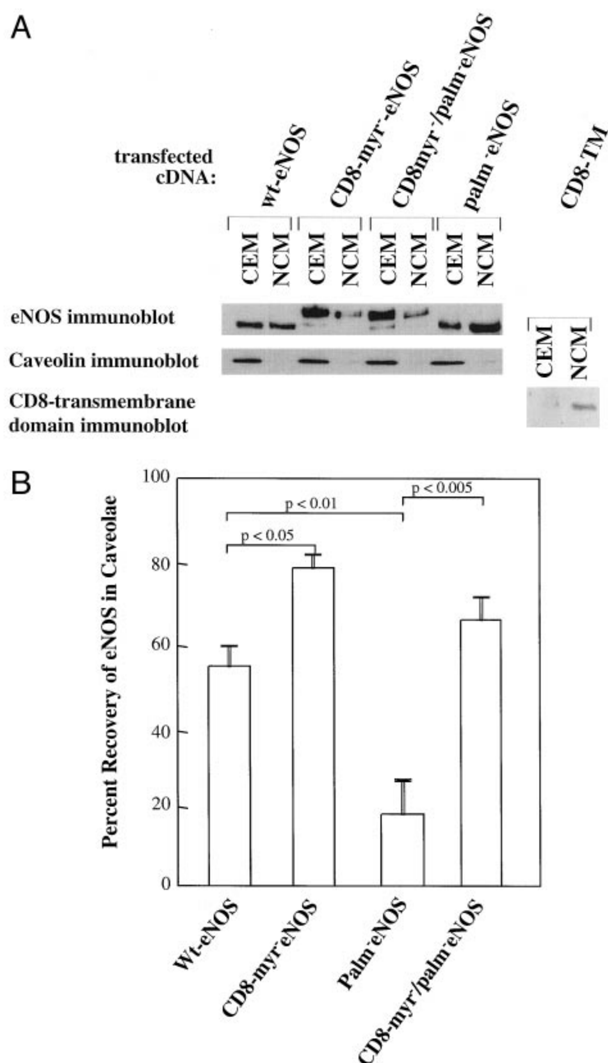

FIG. 4. **Distribution of eNOS chimeras in caveolar and non-caveolar membrane fractions.** Shown are the results of subcellular fractionation and immunoblot analyses of COS-7 cells transfected with cDNA encoding wild-type eNOS (*wt-eNOS*), palm⁻eNOS, CD8-myr⁻eNOS, CD8-myr⁻·palm⁻eNOS, or the CD8 transmembrane 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).

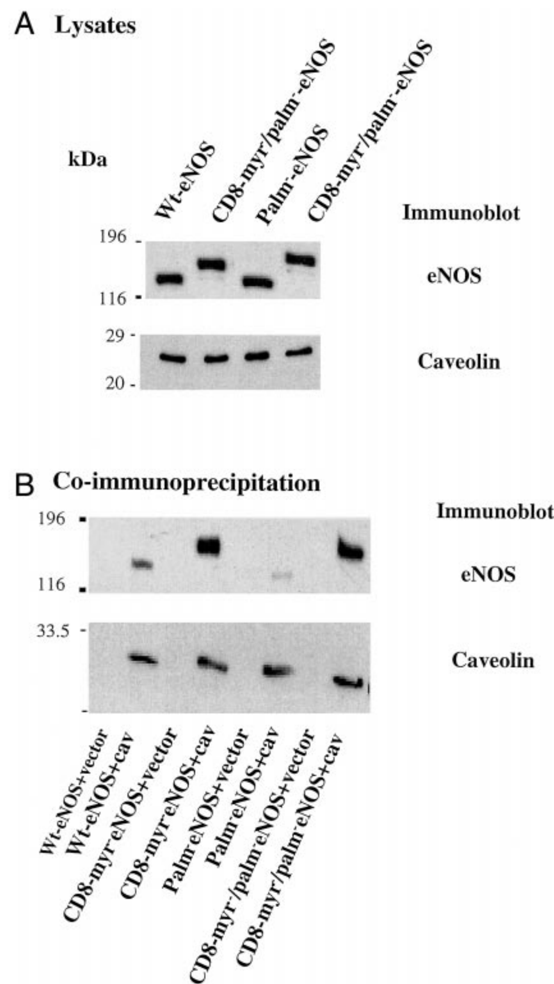

FIG. 5. **Co-immunoprecipitation of caveolin and eNOS chimeras.** Shown are results of co-immunoprecipitation/immunoblot experiments in COS-7 cells co-transfected with cDNA encoding Myc-epitope-tagged caveolin-1 plus either wild-type eNOS (*wt-eNOS*), palm⁻eNOS, CD8-myr⁻eNOS, or CD8-myr⁻·palm⁻eNOS proteins, 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.

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-

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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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REFERENCES

1. Resh, M. (1994) *Cell* **76,** 411–413
2. Milligan, G., Parenti, M., and Magee, A. (1995) *Trends Biochem. Sci.* **20,** 181–187
3. Casey, P. (1995) *Science* **268,** 221–225
4. Wedegaertner, P., Wilson, P., and Bourne, H. (1995) *J. Biol. Chem.* **270,** 503–506
5. Duncan, J., and Gilman, A. (1998) *J. Biol. Chem.* **273,** 15830–15837
6. Yeh, D., Duncan, J., Yamashita, S., and Michel, T. (1999) *J. Biol. Chem.* **274,** 33148–33154
7. Mumby, S. (1997) *Curr. Opin. Cell Biol.* **9,** 148–154
8. Loscalzo J., and Welch G. (1995) *Prog. Cardiovasc. Dis.* **38,** 87–104
9. Michel, T., and Feron, O. (1997) *J. Clin. Invest.* **100,** 146–152
10. Robinson, L., and Michel, T. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 11776–11780
11. Busconi, L., and Michel, T. (1993) *J. Biol. Chem.* **268,** 8410–8413
12. Kantor, D., Lanzrein, M., Stary, S., Sandoval, G., Smith, W., Sullivan, B., Davidson, N., and Schuman, E. (1996) *Science* **274,** 1744–1748
13. Robinson, L., Busconi, L., and Michel, T. (1995) *J. Biol. Chem.* **270,** 995–998
14. Song, S., Li, S., Okamoto, T., Quilliam, L., Sargiacomo, M., and Lisanti, M. (1996) *J. Biol. Chem.* **271,** 9690–9697
15. Bilderback, T., Grigsby, R., and Dobrowsky, R. (1997) *J. Biol. Chem.* **272,** 10922–10927
16. Bilderback, T., Gazula, V., Lisanti, M., and Dobrowsky, R. (1999) *J. Biol. Chem.* **274,** 257–263
17. Feron, O., Belhassen, L., Kobzik, L., Smith, T., Kelly, R., and Michel, T. (1996) *J. Biol. Chem.* **271,** 22810–22814
18. Michel, J., Feron, O., Sase, K., Prabhakar, P., and Michel, T. (1997) *J. Biol. Chem.* **272,** 25907–25912
19. Michel, J., Feron, O., Sacks, D., and Michel, T. (1997) *J. Biol. Chem.* **272,** 15583–15586
20. Bredt, D., and Snyder, S. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 682–685
21. Feron, O., Michel, J., Sase, K., and Michel, T. (1998) *Biochemistry* **37,** 193–200
22. Feron, O., Saldana, F., Michel, J., and Michel, T. (1998) *J. Biol. Chem.* **273,** 3125–3128
23. Dunphy, J., and Linder, M. (1998) *Biochim. Biophys. Acta* **1436,** 245–261
24. Shaul, P., Smart, E., Robinson, L., German, Z., Yuhanna, I., Ying, Y., Anderson, R., and Michel, T. (1996) *J. Biol. Chem.* **271,** 6518–6522
25. Couet, J., Li, S., Okamoto, T., Ikezu, T., and Lisanti, M. P. (1997) *J. Biol. Chem.* **272,** 6525–6533

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