

Depalmitoylation of Endothelial Nitric-oxide Synthase by Acyl-protein Thioesterase 1 Is Potentiated by Ca^{2+} -Calmodulin*

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Protein palmitoylation represents an important mechanism governing the dynamic subcellular localization of many signaling proteins. Palmitoylation of endothelial nitric-oxide synthase (eNOS) promotes its targeting to plasmalemmal caveolae; agonist-promoted depalmitoylation leads to eNOS translocation. Depalmitoylation and translocation of eNOS modulate the agonist response, but the pathways that regulate eNOS palmitoylation and depalmitoylation are poorly understood. We now show that the newly characterized acyl-protein thioesterase 1 (APT1) regulates eNOS depalmitoylation. Immunoblot analyses indicate that APT1 is expressed in bovine aortic endothelial cells, which express eNOS. APT1 overexpression appears to accelerate the depalmitoylation of eNOS in COS-7 cells cotransfected with eNOS and APT1 cDNAs. Additionally, purified recombinant APT1 depalmitoylates eNOS assayed in biological membranes isolated from endothelial cells biosynthetically labeled with [³H]palmitate or COS-7 cells transfected with eNOS cDNA. More important, the APT1-catalyzed depalmitoylation of palmitoyl-eNOS is potentiated by Ca^{2+} -calmodulin (CaM), a key allosteric activator of eNOS. In contrast, APT1-catalyzed depalmitoylation of the G protein $\text{G}\alpha_s$ is unaffected by Ca^{2+} -CaM. Furthermore, caveolin, a palmitoylated membrane protein, does not appear to be a substrate for APT1. Taken together, these results support a role for APT1 in the regulation of eNOS depalmitoylation and suggest that Ca^{2+} -CaM activation of eNOS renders the enzyme more susceptible to APT1-catalyzed depalmitoylation.

Nitric oxide is a key intercellular messenger molecule involved in diverse biological processes and is synthesized in mammalian cells by a family of Ca^{2+} -calmodulin (CaM)¹-activated nitric-oxide synthases (for review, see Ref. 1). The endo-

thelial isoform of nitric-oxide synthase (eNOS) plays a critical role in controlling vascular tone and platelet aggregation. Among the nitric-oxide synthase isoforms, eNOS is unique in its membrane localization. The membrane association of eNOS is conferred by *N*-myristoylation at Gly² and by thiopalmitoylations at Cys¹⁵ and Cys²⁶ (2). *N*-Myristoylation involves cleavage of the N-terminal Met residue and attachment of the 14-carbon unsaturated fatty acid myristic acid to Gly² via an acyl-amide bond. Myristoylation is a cotranslational, usually irreversible process that is catalyzed by a well characterized *N*-myristoyltransferase (for review, see Ref. 3). Palmitoylation represents a distinct type of acylation in which the 16-carbon unsaturated fatty acid palmitic acid is post-translationally attached to the thiol group of a specific Cys residue via an acyl-thioester bond (for review, see Ref. 4). In contrast to the stable acyl-amide bond in *N*-myristoylation, the chemical lability of the thioester bond allows the existence of regulated cycles of palmitoylation and depalmitoylation that may control a protein's subcellular localization.

One critical function of protein palmitoylation appears to be tethering otherwise soluble proteins to the plasmalemmal membrane. Indeed, although *N*-myristoylated and prenylated signaling proteins are found in the cytoplasm as well as on cellular membranes, palmitoylation of signaling proteins confines them to cellular membranes (for review, see Ref. 4). Moreover, for several signaling proteins (including Src-like tyrosine kinases (5), the heterotrimeric G protein α subunit $\text{G}\alpha_{11}$ (6), and eNOS (7)), palmitoylation targets the protein to specific signal transduction microdomains in the plasmalemmal membrane termed caveolae. Cycles of protein palmitoylation and depalmitoylation may control a protein's distribution between membrane and cytoplasm and/or between subdomains of the plasma membrane (e.g. caveolae versus non-caveolae) and may modulate the coupling of specific signaling proteins to either receptors or intracellular effectors. To date, four examples of agonist-regulated protein depalmitoylation have been reported: the β_2 -adrenergic receptor (8), the m_2 muscarinic acetylcholine receptor (9), the heterotrimeric G protein α subunit $\text{G}\alpha_s$ (10), and eNOS (11). However, the mechanisms by which activation-dependent changes in these proteins' palmitoylation state are achieved remain elusive.

Numerous studies have attempted to identify protein palmitoyltransferases that palmitoylate proteins and protein palmitoylthioesterases that depalmitoylate proteins, but these efforts to date have been mostly unsuccessful. A cytosolic protein palmitoylthioesterase termed acyl-protein thioesterase 1 (APT1) has been recently purified and cloned from rat liver: APT1 catalyzes the depalmitoylation of heterotrimeric G protein α subunits and p21^{ras} (12). APT1 was originally isolated from rat liver as a lysophospholipase that hydrolyzes lysophosphatidylcholine into saturated fatty acid and *sn*-glycero-3-

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¹ The abbreviations used are: CaM, calmodulin; eNOS, endothelial nitric-oxide synthase; APT1, acyl-protein thioesterase 1; BAEC, bovine aortic endothelial cell(s); PAGE, polyacrylamide gel electrophoresis; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; CAPSO, 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonic acid.

phosphocholine (13). However, Duncan and Gilman (12) have demonstrated that 1) the K_m of APT1 for acyl-protein substrates is 250-fold lower than the respective values observed for lysophosphatidylcholine and that 2) APT1 can act *in situ* to depalmitoylate G_{α_s} when coexpressed in HEK293 cells. The evidence presented by Duncan and Gilman suggests that APT1 may represent the first authentic participant in regulated depalmitoylation of intracellular signaling proteins.

This study investigates whether APT1 is also a physiological regulator of eNOS depalmitoylation. To this end, it is important to address several issues. First, is APT1 endogenously expressed in eNOS-expressing cells (*e.g.* endothelial cells)? Second, can coexpression of APT1 with eNOS in a heterologous mammalian system accelerate the rate of eNOS depalmitoylation *in situ*? Third, can purified APT1 depalmitoylate eNOS *in vitro*? Finally, if APT1 does appear to depalmitoylate eNOS, does APT1 have a preference for the activated form of eNOS over the nonactivated form of eNOS?

EXPERIMENTAL PROCEDURES

Plasmid Constructs—For eukaryotic expression of rat APT1, APT1 cDNA (13) was cloned into the pBK-CMV (Stratagene) or pcDNA3 (Invitrogen) vector. For purification of APT1 from a prokaryotic expression system, His₆-tagged rat APT1 cDNA in the bacterial expression vector pQE60 (QIAGEN Inc.) was used as described previously (12). A mammalian expression vector encoding eNOS (pK-ENH) has been previously described (11). Plasmid constructs for expression of G_{α_s} in *Escherichia coli* have also been described (14). Mutants of eNOS with residues 10–14 deleted (termed eNOS^{S10–14}), with residues 18–25 deleted (termed eNOS^{GL1}), and with residues 16–25 all converted into Gly (termed eNOS^{G10}) (see Fig. 7) were generated by polymerase chain reaction-based mutagenesis employing oligonucleotides that encoded site-specific mutations or deletions as primary primers and pK-ENH as a template. Secondary primers were then used with the previously generated fragments as templates to create 850-base pair mutation-encoding fragments starting from –70 base pairs and ending at 780 base pairs. The purified polymerase chain reaction products of the second polymerase chain reaction were digested with *Bam*HI and *Eco*RI for unidirectional ligation into reciprocally digested pK-ENH. The constructs were restriction-mapped to confirm correct insertion, and the sequences of the polymerase chain reaction fragments were verified by dideoxynucleotide sequencing.

Cell Culture and Transfection—Bovine aortic endothelial cells (BAEC; obtained from Cell Systems Corp.) were cultured as described previously (15). Cells were used between passages 5 and 9. The COS-7 cell line was grown and transfected with 15–30 μ g of DNA by electroporation (BTX Electro Cell Manipulator). For cotransfections, an equivalent amount of each plasmid was transfected for a total of 30 μ g of DNA. The nitric-oxide synthase activities of wild-type and mutant eNOS were determined by measuring the conversion of radiolabeled L-[³H]arginine to L-[³H]citrulline and nitric oxide as described by Bredt and Schmidt (16).

Biosynthetic Radiolabeling—Cultures were radiolabeled with [³H]palmitic acid (40–60 Ci/mmol) by incubation of cells in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) containing 10% dialyzed fetal bovine serum (Hyclone Laboratories), 2% penicillin/streptomycin (Life Technologies, Inc.), and 1 mCi/ml (~20 μ M) [³H]palmitate (NEN Life Science Products) for 2 h at 37 °C.

Preparation of Cell Lysates and Subcellular Fractionation—Transfected COS-7 cells or BAEC were harvested by scraping in phosphate-buffered saline, pelleted by centrifugation, and then resuspended in either Buffer A (10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate, 100 mM NaCl, 2 mM dithiothreitol, and 2 μ g/ml each antipain, leupeptin, lima bean trypsin inhibitor, and soybean trypsin inhibitor) or Buffer B (50 mM Hepes (pH 7.5), 10 mM NaCl, 5 mM MgCl₂, 0.1 mM EDTA, 5 mM dithiothreitol, 0.5 mM L-arginine, 10 μ M BH₄, and protease inhibitors as listed for Buffer A). Cells were lysed by sonication using a Branson Model 450 sonifier; cell debris was discarded following a brief 1000 \times g centrifugation. When subcellular fractionation was required, cells were lysed in Buffer B and separated into soluble and particulate fractions by ultracentrifugation (100,000 \times g) for 1 h. Protein concentration was determined using the Bradford reagent (Bio-Rad).

Assessment of Depalmitoylation of eNOS by APT1 in Transfected COS-7 Cells—To examine the effects of APT1 on eNOS depalmitoyla-

tion *in situ*, pulse-chase experiments were performed in COS-7 cells coexpressing eNOS and APT1 or eNOS and a control plasmid (pBK-CMV). After labeling transfected COS-7 cells with 1 mCi/ml [³H]palmitate for 2 h, cultures were washed with Dulbecco's modified Eagle's medium containing 10% dialyzed fetal bovine serum and 2% penicillin/streptomycin with 100 μ M unlabeled palmitate and then incubated in the same chase medium for the indicated durations. Cells were harvested in Buffer A as described above and immunoprecipitated as described below.

Purification of APT1 from *E. coli*—To produce purified recombinant His₆-tagged APT1, cultures of *E. coli* were grown; protein expression was induced; and soluble lysate was prepared as described previously (12). The soluble lysate was then loaded onto a Ni²⁺-nitrilotriacetic acid-agarose column (QIAGEN Inc.). The column was washed with 5 column volumes of 50 mM Tris-HCl (pH 8.0) supplemented with 100 mM NaCl and 10 mM imidazole (Sigma). The protein was eluted from the column with 50 mM Tris-HCl (pH 8.0) supplemented with 100 mM imidazole. The purified protein was concentrated in a Centrprep-10 (Millipore Corp.) and stored at –80 °C.

Preparation of Palmitoylated G_{α_s} —Recombinant heterotrimeric G_s was prepared by combining purified G_{α_s} (produced in *E. coli*) with purified $G\beta_1\gamma_2$ (from recombinant baculovirus-infected Sf9 cells) at a 1:1 molar ratio. Heterotrimeric G_s (10 μ M) was incubated at 30 °C for 30 min with [³H]palmitoyl-CoA (~1000 cpm/pmol, 100 μ M) as described previously by Duncan and Gilman (17). Incorporation of [³H]palmitate into G_{α_s} , but not $G\beta_1$ or $G\gamma_2$, was confirmed by SDS-PAGE and fluorography. Excess palmitoyl-CoA was removed from the sample by gel filtration chromatography using Sephadex G-50 (Amersham Pharmacia Biotech), and palmitoylated heterotrimeric G_s was concentrated to ~1 μ M (assessed by liquid scintillation spectrometry) and stored at –80 °C until used in depalmitoylation assays.

Assessment of *In Vitro* Depalmitoylation of eNOS, eNOS Mutants, Caveolin, and G_{α_s} by Purified APT1—To examine the ability of purified recombinant APT1 to depalmitoylate eNOS, the eNOS mutants, and caveolin *in vitro*, COS-7 cells transfected with eNOS- or eNOS mutant-expressing plasmids and BAEC expressing endogenous eNOS and caveolin were used. Two days post-transfection, COS-7 cells were labeled with 1 mCi/ml [³H]palmitate and harvested in Buffer B. BAEC were labeled when confluent and harvested in Buffer B. Cell lysates were sonicated (two 10-s bursts), and the particulate fraction obtained after microcentrifugation (maximum speed for 30 min) was resuspended in Buffer B with a 27-gauge needle. Glycerol was added to a final concentration of 10%, and protein concentration was determined with the Bradford reagent. Protein from either the soluble or particulate fraction (or purified [³H]palmitoyl- G_{α_s}) was incubated at 30 °C with purified APT1 in APT1 reaction buffer (50 mM Hepes (pH 8.0), 2 mM MgCl₂, 0.1 mM EDTA, 7.5 mM CHAPS, 0.5 mM L-arginine, 10 μ M BH₄, 6 μ g/ml leupeptin, 6 μ g/ml lima bean trypsin inhibitor, 32 μ g/ml L-1-tosylamido-2-phenylethyl chloromethyl ketone, and 32 μ g/ml N^ε-p-tosyl-L-lysine chloromethyl ketone) in a total volume of 1 ml. At specified time points, 100 μ l of the reaction was removed and placed in 900 μ l of ice-cold Buffer A supplemented with 1 mM phenylmethylsulfonyl fluoride to terminate the APT1 reaction. Samples were then immunoprecipitated (except for those assaying purified [³H]palmitoyl- G_{α_s}) and processed as described below. The effects of Ca²⁺-CaM (Calbiochem) and EGTA (Sigma) were assessed by adding the reagent to the reaction immediately before adding APT1.

Immunoprecipitation—Immunoprecipitation of eNOS was done in Buffer A using a polyclonal antiserum against eNOS as described previously (18). Immunoprecipitation of caveolin was done using 4 μ g/ml polyclonal anti-caveolin antibody (Transduction Laboratories) according to the manufacturer's protocol. Immunoprecipitates were eluted from protein A-Sepharose with SDS-PAGE sample buffer containing either 5 mM dithiothreitol (for [³H]palmitate-labeled samples) or 5% β -mercaptoethanol (for all other samples).

SDS-PAGE, Autoradiography, and Immunoblotting—Proteins were analyzed by SDS-PAGE and electrotransferred to polyvinylidene difluoride membranes (Bio-Rad) in 25 mM CAPSO (pH 10) and 20% methanol. Polyvinylidene difluoride membranes with radiolabeled proteins were analyzed by the Cyclone Storage PhosphorImager (Packard Instrument Co.); signals were quantitated using Optiquant System software (Packard Instrument Co.). For immunoblotting of eNOS and caveolin, monoclonal antibodies directed against eNOS or caveolin (Transduction Laboratories) were used for chemiluminescent detection of proteins as described previously (19). Immunoblotting of G_{α_s} was done with antiserum 584 (20). For immunoblotting of APT1, membranes were blocked with 5% nonfat dry milk in Tris-buffered saline with 0.1% (v/v) Tween 20 (TBST) and incubated with a previously

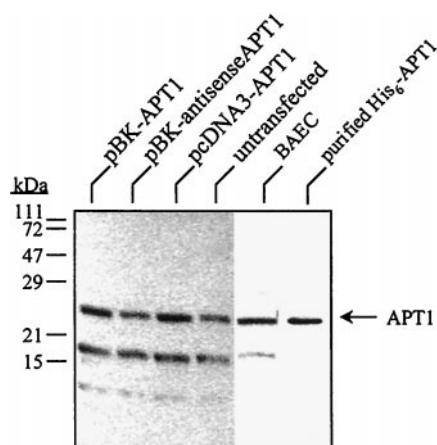


FIG. 1. APT1 protein expression in endothelial cells. Shown is an immunoblot probed with the anti-APT1 antiserum. Cell lysates (first through fifth lanes) and purified APT1 protein (sixth lane) were resolved by SDS-PAGE on an 18% gel, transferred to polyvinylidene difluoride, and immunoblotted, and immunoreactive proteins were detected by chemiluminescence as described under "Experimental Procedures." The first four lanes show COS-7 cells transfected with the APT1-expressing plasmid pBK-APT1, a COS-7 cell negative control (transfected with pBK-antisenseAPT1), COS-7 cells transfected with the APT1-expressing plasmid pcDNA3-APT1, and untransfected COS-7 cells, respectively. The fifth lane shows the results of an immunoblot of BAEC lysates. The sixth lane contains recombinant APT1 purified from *E. coli*. This experiment was repeated three times with similar results.

described anti-APT1 antiserum (13) for 1 h in TBST containing 1% nonfat dry milk. The anti-APT1 antiserum was titrated between 1:100 and 1:10,000 to determine optimal conditions. After three washes (5 min each), the membranes were incubated for 1 h with horseradish peroxidase-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc.) at a 1:20,000 dilution in TBST containing 1% nonfat dry milk. After three additional washes in TBST, the membranes were incubated with a chemiluminescent reagent (Pierce) according to the manufacturer's protocols, exposed to x-ray film, and quantitated by densitometry or directly quantitated using the ChemImager 4000 (Alpha Innotech). eNOS protein abundance was quantitated in the linear range of chemiluminescence detection using ChemImager software (Alpha Innotech) and was validated over a range of protein concentrations. Determinations of labeling half-life applied the equation for single exponential decay, and the statistical significance of the derived values was evaluated by analysis of variance using GraphPAD Prism software.

RESULTS

Endogenous Expression of APT1 in Bovine Aortic Endothelial Cells—Of first importance in assessing the possibility of eNOS depalmitoylation by APT1 was determining whether cells that characteristically express eNOS also express APT1. Lysates of BAEC (cells known to express eNOS robustly (15)) were analyzed by immunoblots probed with a previously described polyclonal antiserum directed against APT1 (13). In BAEC, the anti-APT1 antiserum (but not nonimmune serum) detected a major immunoreactive band (presumably APT1) at the predicted molecular mass of 25 kDa (Fig. 1, fifth lane); this band is identical in molecular mass to that of pure APT1 (sixth lane; purified to homogeneity on a Ni^{2+} -nitrilotriacetic acid column following expression of His₆-tagged APT1 in *E. coli* as described under "Experimental Procedures"). An additional immunoreactive protein was seen in BAEC and COS-7 cells at ~18 kDa; this band may represent either an irrelevant immunoreactive protein or a degradation product of APT1. COS-7 cells were transfected with APT1-expressing plasmids (pBK-APT1 and pcDNA3-APT1) or a control plasmid (pBK-antisenseAPT1) or were not transfected (*untransfected*, as shown); cell lysates were immunoblotted with the anti-APT1 antiserum. The immunodetection of greater quantities of the 25-kDa protein in cells transfected with APT1-expressing plasmids suggests that

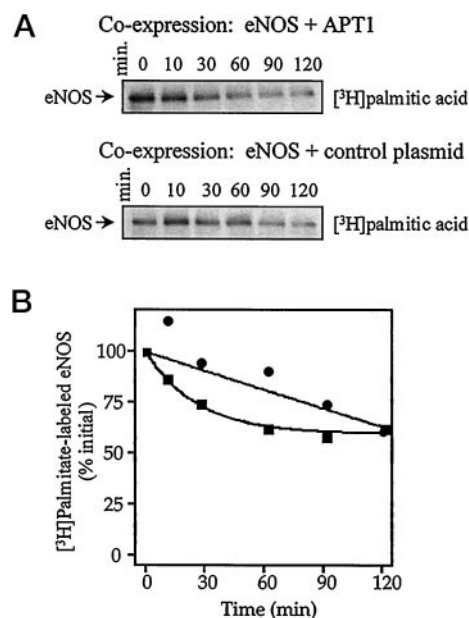


FIG. 2. In situ depalmitoylation of eNOS in COS-7 cells cotransfected with APT1 cDNA. COS-7 cells were cotransfected with plasmids expressing eNOS and APT1 (■) or eNOS and control plasmid (●) and biosynthetically labeled with [³H]palmitate for 2 h. Radiolabeled cells were washed twice in medium containing 100 μM unlabeled palmitate and incubated in medium containing 100 μM unlabeled palmitate and harvested at the indicated times. eNOS was immunoprecipitated from cell lysates, analyzed by SDS-PAGE, electroblotted, and exposed on a tritium-sensitive PhosphorImager screen. A, shown is [³H]palmitate remaining on eNOS after the indicated chase times. Equal amounts of protein were loaded per lane. B, the data are fit to the equation for single exponential decay. This experiment was performed three times with equivalent results.

the 25-kDa band is indeed APT1 (Fig. 1). The APT1 constructs in pBK-CMV and the pcDNA3 vectors yielded approximately equivalent amounts of recombinant APT1 expression, approximately double the endogenous APT1 level seen in control COS-7 cells.

Depalmitoylation of eNOS by APT1 in Transfected COS-7 Cells—To test whether APT1 affects the turnover rate of [³H]palmitate on eNOS, COS-7 cells were cotransfected with cDNA constructs encoding eNOS plus either an APT1-expressing plasmid or a control plasmid. Two days after transfection, the cells were incubated in medium containing 1 mCi/ml [³H]palmitic acid and harvested at various times, and eNOS was immunoprecipitated from cell lysates and analyzed following SDS-PAGE. The rate of biosynthetic incorporation of [³H]palmitate into eNOS was not substantively altered by co-expression of APT1 with eNOS relative to the rate observed when eNOS was coexpressed with a control plasmid (data not shown). However, as shown in Fig. 2, pulse-chase experiments ($n = 3$) revealed a small but reproducible increase in the rate of eNOS depalmitoylation in COS-7 cells cotransfected with eNOS plus APT1 cDNAs. This modest (but consistent) effect of APT1 cotransfection on eNOS depalmitoylation may reflect the low level of APT1 overexpression achieved in transient transfection experiments using these constructs, confounded further by the fairly robust endogenous expression of APT1 in control COS-7 cells. Therefore, for more detailed characterizations, we sought to further characterize the effects of APT1 by experiments in cell-free systems using purified protein.

Depalmitoylation of eNOS by Purified APT1—Recombinant His₆-tagged APT1 expressed in *E. coli* was purified to >95% purity in a single Ni^{2+} -nitrilotriacetic acid affinity column chromatography step (see Fig. 1) (12) and used to study the depalmitoylation of eNOS in biological membranes isolated

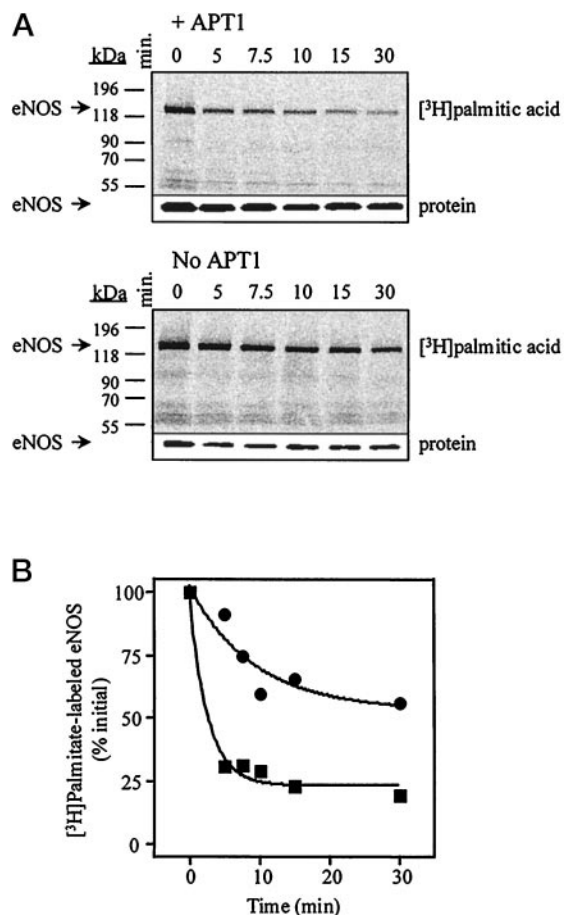


FIG. 3. eNOS depalmitoylation by purified APT1. Crude membrane fractions were prepared from COS-7 cells that had been transfected with eNOS and biosynthetically labeled with [^3H]palmitate. 50 μg of membrane protein was incubated with either 10 μg of purified APT1 (+ APT1, \blacksquare) or an equal volume of reaction buffer (No APT1, \bullet) for the indicated times. *A*, eNOS was immunoprecipitated, analyzed by SDS-PAGE, electroblotted, and exposed on a tritium-sensitive PhosphorImager screen as described under "Experimental Procedures." Beneath each screen showing results obtained for analysis of [^3H]palmitate labeling is an immunoblot of the same filter probed with the anti-eNOS antibody and analyzed by chemiluminescence. *B*, the data are fit to the equation for single exponential decay. The amount of initial [^3H]palmitate remaining on eNOS was determined after normalizing to the amount of protein present. The experiment was repeated five times with similar results.

from either BAEC or eNOS-transfected COS-7 cells that had been biosynthetically labeled with [^3H]palmitate. As shown in Fig. 3, purified APT1 significantly accelerated eNOS depalmitoylation: in the presence of APT1, there was a marked decrease in [^3H]palmitate labeling of eNOS, with no substantive change in the total eNOS protein quantitated by immunoblotting. The half-life of [^3H]palmitate-labeled eNOS decreased from 14.3 ± 1.6 to 3.1 ± 0.3 min when membranes were incubated in the presence of APT1 (mean \pm S.E. for $n =$ three independent experiments; $p < 0.001$); there was no change in the half-life of the eNOS protein itself. Boiling APT1 completely abrogated its ability to potentiate the depalmitoylation of eNOS (data not shown). Endogenous eNOS biosynthetically labeled with [^3H]palmitate in BAEC and recombinant wild-type eNOS in transfected COS-7 cells were indistinguishable with respect to their ability to be depalmitoylated by purified APT1.

Duncan and Gilman (12) have provided support for activation-dependent regulation of the depalmitoylation of $\text{G}\alpha_s$ by demonstrating that when $\text{G}\alpha_s$ is used as a substrate for APT1 *in*

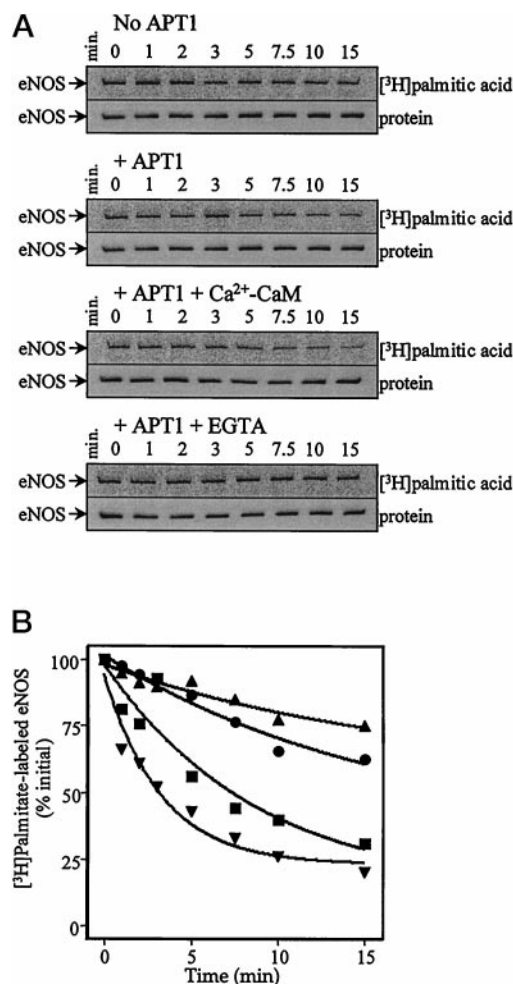


FIG. 4. eNOS depalmitoylation by purified APT1: opposing effects of Ca^{2+} -CaM and EGTA. As described in the legend to Fig. 3, crude membrane fractions were prepared from COS-7 cells that had been transfected with eNOS and biosynthetically labeled with [^3H]palmitate. 50 μg of protein from the labeled membrane fraction was then incubated for the specified times with 10 μg of purified APT1 (+ APT1, \blacksquare), an equal volume of reaction buffer (No APT1, \bullet), or 10 μg of purified APT1 plus 1.2 mM CaCl_2 and 10 μM CaM (+ APT1 + Ca^{2+} -CaM, \blacktriangledown) or 5 mM EGTA (+ APT1 + EGTA, \blacktriangle). *A*, eNOS was immunoprecipitated, analyzed by SDS-PAGE and electroblotting, and then exposed on a tritium-sensitive PhosphorImager screen as described under "Experimental Procedures." Beneath each screen showing results obtained for analysis of [^3H]palmitate labeling is an immunoblot of the same filter probed with the anti-eNOS antibody and analyzed by chemiluminescence. *B*, the graph represents average values of three to four experiments. Error bars represent S.E. The data are fit to the equation for single exponential decay. The amount of initial [^3H]palmitate remaining on eNOS was determined after normalizing to the amount of protein present.

vitro, the activated dissociated α subunit is depalmitoylated by APT1 more rapidly than is the inactive heterotrimer. We have previously found that agonist activation of eNOS in bovine aortic endothelial cells can promote the enzyme's depalmitoylation *in situ* (11). To explore whether APT1 depalmitoylation of eNOS might be affected by the activation of eNOS *in vitro*, we studied the effects of Ca^{2+} -CaM, a key allosteric activator of eNOS (reviewed in Ref. 21). As shown in Fig. 4, the addition of Ca^{2+} -CaM to incubations of [^3H]palmitoyl-eNOS with APT1 potentiated the depalmitoylation of eNOS relative to incubations with APT1 alone: the $t_{1/2}$ of [^3H]palmitoyl-eNOS decreased from 3.1 ± 0.3 to 2.3 ± 0.2 min with the addition of Ca^{2+} -CaM (mean \pm S.E. for $n =$ three independent experiments; $p < 0.04$). In the absence of APT1, the addition of Ca^{2+} -CaM did not increase the rate of eNOS depalmitoylation over that occurring

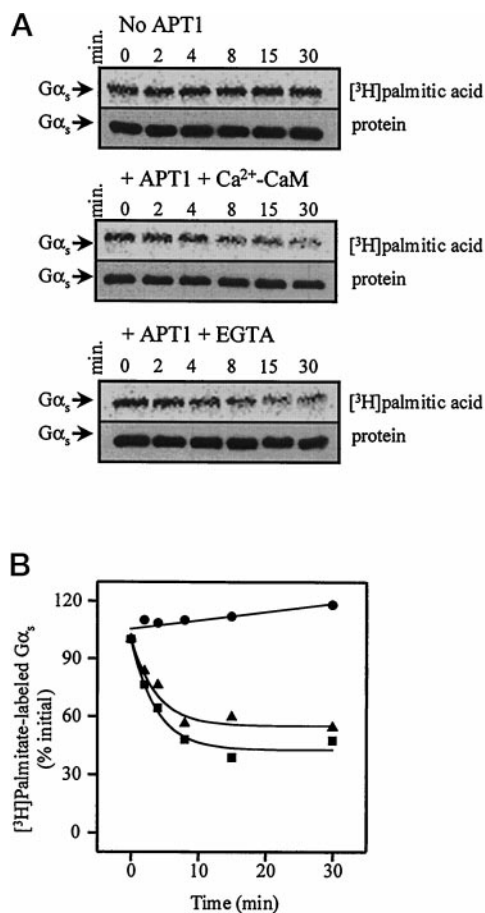


FIG. 5. Depalmitoylation of G α_s by APT1: no effect of Ca $^{2+}$ -CaM or EGTA. 3 H-Palmitoylated G α_s was prepared by autoacylation of heterotrimeric G α_s as described under "Experimental Procedures." The G α_s substrate (50 nM) was incubated for the specified times with reaction buffer (No APT1, ●), 600 pg/ μ l purified APT1 plus 1.2 mM CaCl $_2$ and 10 μ M CaM (+ APT1 + Ca $^{2+}$ -CaM, ▲), or 600 pg/ μ l purified APT1 plus 5 mM EGTA (+ APT1 + EGTA, ■). A, [3 H]palmitate associated with G α_s was assessed by SDS-PAGE followed by electroblotting and exposure to a tritium-sensitive PhosphorImager screen. Shown below each image of radioactive [3 H]palmitate is an immunoblot of the same filter probed with antisera directed against G α_s . B, the graph represents quantitative analysis of the data presented in A carried out as described in the legend to Fig. 4. The data are fit to the equation for single exponential decay. The experiment is representative of two independent experiments.

in buffer alone (data not shown). As shown in Fig. 4, the addition of EGTA, a Ca $^{2+}$ chelator, was found consistently to attenuate APT1-catalyzed eNOS depalmitoylation: the $t_{1/2}$ of [3 H]palmitoyl-eNOS increased from 3.1 ± 0.3 to 4.6 ± 0.9 min with the addition of EGTA (mean \pm S.E. for $n =$ three independent experiments; $p < 0.02$).

To demonstrate the specificity of the effect of Ca $^{2+}$ -CaM and EGTA on APT1-catalyzed depalmitoylation, we explored the effects of these reagents on APT1-promoted depalmitoylation of the G protein G α_s , a signaling protein not known to directly bind Ca $^{2+}$ -CaM. Fig. 5 demonstrates that, in contrast to the opposing effects of Ca $^{2+}$ -CaM and EGTA on APT1 depalmitoylation of eNOS, neither Ca $^{2+}$ -CaM nor EGTA had any effect on APT1 depalmitoylation of G α_s . Thus, in marked contrast to the effects seen with eNOS, the addition of Ca $^{2+}$ -CaM does not significantly potentiate APT1-catalyzed depalmitoylation of G α_s , nor does the Ca $^{2+}$ chelator EGTA inhibit APT1-promoted depalmitoylation of G α_s .

To determine whether APT1 can also promote the depalmitoylation of other palmitoylated proteins, we conducted similarly designed experiments using caveolin, a 22-kDa palmitoy-

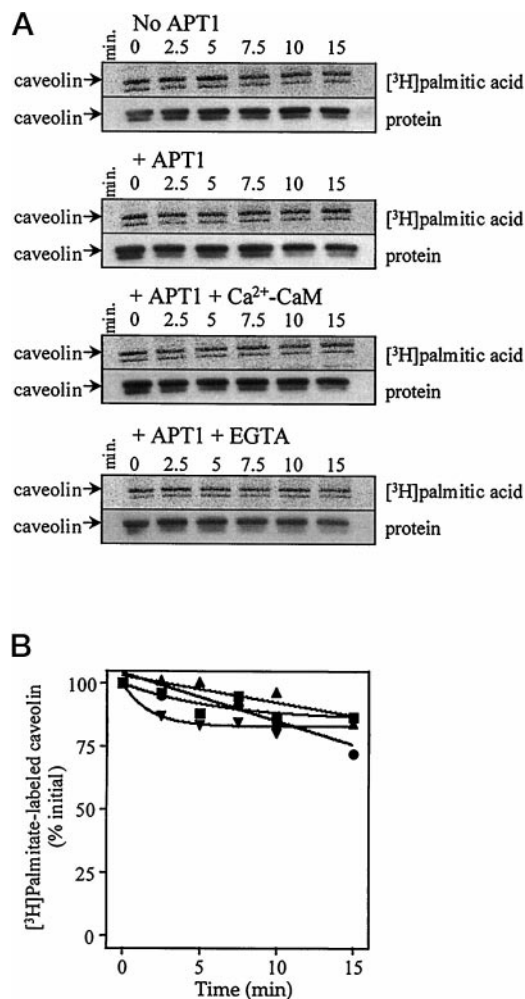


FIG. 6. Caveolin as a substrate for purified APT1. BAEC were biosynthetically labeled with [3 H]palmitate for 2 h and harvested. 50 μ g of protein from the labeled membrane fraction was then incubated for the specified times with 10 μ g of purified APT1 (+ APT1, ■), an equal volume of reaction buffer (No APT1, ●), or 10 μ g of purified APT1 plus 1.2 mM CaCl $_2$ and 10 μ M CaM (+ APT1 + Ca $^{2+}$ -CaM, ▼) or 5 mM EGTA, a Ca $^{2+}$ chelator (+ APT1 + EGTA, ▲). A, caveolin was immunoprecipitated, analyzed by SDS-PAGE and electroblotting, and then exposed on a tritium-sensitive PhosphorImager screen as described under "Experimental Procedures." Beneath each screen showing results obtained for analysis of [3 H]palmitate labeling is an immunoblot of the same filter probed with the anti-caveolin antibody and analyzed by chemiluminescence. B, the data are fit to the equation for single exponential decay. The amount of initial [3 H]palmitate remaining on caveolin was determined after normalizing to the amount of protein present.

lated integral membrane protein that interacts with and regulates eNOS (22) and other signaling proteins (23). Caveolin biosynthetically labeled with [3 H]palmitate did not appear to be a substrate for APT1 (Fig. 6). Furthermore, the addition of Ca $^{2+}$ -CaM did not trigger APT1 depalmitoylation of caveolin, and EGTA did not significantly alter the lack of depalmitoylating activity of APT1 for caveolin (Fig. 6), again suggesting the specificity of Ca $^{2+}$ -CaM-accelerated APT1 depalmitoylation of eNOS.

To further characterize the sequences in eNOS important to its recognition by APT1, several eNOS mutants were generated with mutations in the sequence between and around Cys 15 and Cys 26 , the two palmitoylated Cys residues of eNOS (Fig. 7). As the two palmitoylated Cys residues are separated by an unusual 10-residue sequence of five tandem Gly-Leu repeats ((Gly-Leu) $_5$), the eNOS GL1 and eNOS G10 mutants explored the effects of spacing and protein flexibility, respectively, between

the two palmitoylated Cys residues (Fig. 7). Another mutant, termed eNOS^{Δ10-14}, was used to explore whether the distance between the myristoylated Gly² and palmitoylated Cys residues affects the ability of eNOS to be depalmitoylated by APT1. All these mutants were expressed at the same level as wild-type eNOS in transfected COS-7 cells (shown by immunoblot analysis; Fig. 7) and had intact nitric-oxide synthase activity (assayed using the L-[³H]arginine to L-[³H]citrulline assay described above; data not shown). The eNOS^{GL1} and eNOS^{G10} mutants, which contain alterations in the amino acid sequence between the two palmitoylated Cys residues, showed no [³H]palmitate incorporation in biosynthetic labeling experiments using COS-7 cells transiently transfected with these cDNAs that express mutant eNOS proteins. However, the deletion mutant eNOS^{Δ10-14} did incorporate [³H]palmitate in biosynthetic labeling experiments; furthermore, purified APT1 promoted the depalmitoylation of the [³H]palmitate-labeled eNOS^{Δ10-14} mutant in biological membranes, as seen for the wild-type eNOS (Fig. 7).

DISCUSSION

Several lines of evidence in this study suggest that APT1 may be a physiological regulator of eNOS depalmitoylation. We have shown that APT1 is expressed in BAEC, cells that also express eNOS (Fig. 1, *fifth lane*). The presence of APT1 in BAEC suggests that, at least with respect to cellular distribution, APT1 has the possibility to interact with eNOS in a physiologically relevant manner. We have also shown that coexpression of APT1 can accelerate eNOS depalmitoylation *in situ* in transiently transfected COS-7 cells (Fig. 2) and that purified APT1 can depalmitoylate eNOS (Fig. 3). The potentiation by Ca²⁺-CaM of APT1-catalyzed eNOS depalmitoylation (Fig. 4) provides a potential regulatory mechanism controlling this key process and suggests that APT1 may indeed be responsible for the agonist-induced depalmitoylation of eNOS seen in BAEC.

In addition to its effects on eNOS, APT1 is able to depalmitoylate heterotrimeric G protein α subunits and p21^{ras} (12). Furthermore, with respect to at least eNOS and heterotrimeric G proteins, APT1 shows a preference for the activated state of the protein. Thus, the same enzyme, APT1, appears able to depalmitoylate structurally dissimilar signaling proteins. APT1 does, however, show some substrate selectivity in that APT1 does not seem to promote the depalmitoylation of caveolin (Fig. 6). Caveolin differs from heterotrimeric G protein α subunits and eNOS in that 1) it is an integral membrane protein; 2) it is palmitoylated near the C terminus rather than the N terminus; and 3) its palmitoylation is not known to be modulated by agonists (24). No consensus sequence has been defined for protein palmitoylation: the only common aspect of palmitoylated Cys residues appears to be that the sites are found in proximity to either other lipid-modified residues or putative transmembrane domains. The seeming promiscuity of palmitoylation is evident not only in the diversity of sequences flanking palmitoylated Cys residues (4), but also in the palmitoylation of both transmembrane proteins (*e.g.* caveolin) and otherwise soluble proteins (*e.g.* eNOS). It will be especially interesting to test whether integral membrane proteins such as the β_2 -adrenergic receptor and the m₂ muscarinic acetylcholine receptor (both of which exhibit agonist-modulated depalmitoylation) are also substrates for APT1.

Although a consensus sequence for protein palmitoylation has yet to be determined, there are illustrative features of the eNOS mutants in which the sequence between the palmitoylated cysteines (Cys¹⁵ and Cys²⁶) are altered: palmitoylation is abrogated when the intervening 10-amino acid sequence (Gly-Leu)₅ is shortened to Gly-Leu or replaced by Gly₁₀ (all five Leu

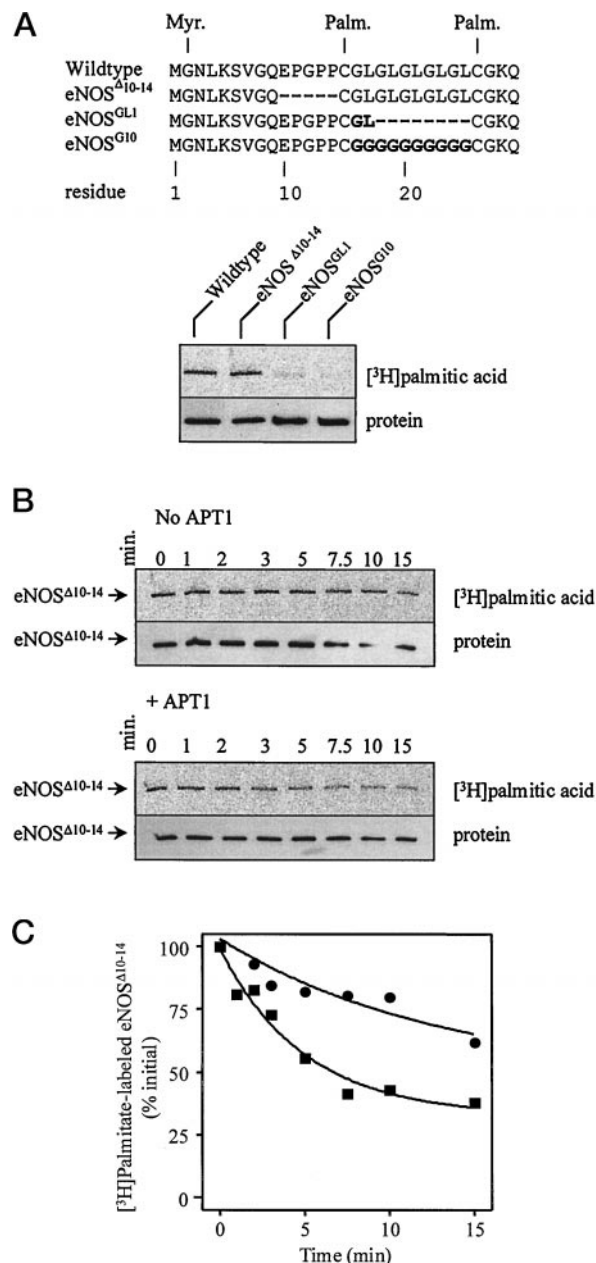


Fig. 7. Mutagenesis of residues near the eNOS palmitoylation sites: effects on depalmitoylation by APT-1. COS-7 cells were transfected with various eNOS mutants as shown and then biosynthetically labeled with [³H]palmitate and harvested. **A**, shown are the sequences of wild-type eNOS and the eNOS^{Δ10-14}, eNOS^{GL1}, and eNOS^{G10} mutants. The *lower panel* shows the eNOS mutants after immunoprecipitation, SDS-PAGE, electroblotting, and exposure on a tritium-sensitive PhosphorImager screen as described under "Experimental Procedures." Beneath each screen showing results obtained for analysis of [³H]palmitate labeling is an immunoblot of the same filter probed with the anti-eNOS antibody and analyzed by chemiluminescence. **B**, 50 μ g of protein from the [³H]palmitate-labeled membrane fraction of COS-7 cells transfected with the eNOS^{Δ10-14} mutant was incubated with 10 μ g of purified APT1 (+ APT1, \blacksquare) or an equal volume of reaction buffer (No APT1, \bullet) for the indicated times. The eNOS^{Δ10-14} mutant was then immunoprecipitated, analyzed by SDS-PAGE and electroblotting, and then exposed on a tritium-sensitive PhosphorImager screen as described under "Experimental Procedures." Beneath each screen showing results obtained for analysis of [³H]palmitate labeling is an immunoblot of the same filter probed with the anti-eNOS antibody and analyzed by chemiluminescence. **C**, the data are fit to the equation for single exponential decay. The amount of initial [³H]palmitate remaining on eNOS was determined after normalizing to the amount of protein present. This experiment was repeated three times with equivalent results. Myr., myristoylation site; Palm., palmitoylation sites.

residues changed to Gly). By contrast, deletion of a five-amino acid sequence located immediately N-terminal to Cys¹⁵ has no effect on palmitoylation in cells or depalmitoylation by APT1.

Membrane-associated eNOS likely represents the physiological substrate for APT1, and therefore, [³H]palmitoyl-eNOS in biological membranes was used as a substrate to explore the potential role for APT1 in eNOS depalmitoylation. Purified recombinant APT1 can depalmitoylate [³H]palmitoyl-eNOS derived from membranes of BAEC and eNOS-transfected COS-7 cells biosynthetically labeled with [³H]palmitate. Although experiments under these *in vitro* conditions may be considered less physiological than *in situ* coexpression experiments, the use of purified APT1 allowed the unambiguous identification of APT1 as the agent responsible for the ~4-fold acceleration of eNOS depalmitoylation (Fig. 3). The fact that we observed positive effects of APT1 on eNOS depalmitoylation in biological membranes helps to validate this approach. Adding further physiological credibility to the *in vitro* demonstration of APT1 depalmitoylation of eNOS is our observation that APT1-catalyzed depalmitoylation of eNOS can be regulated specifically by compounds that affect the eNOS palmitoylation state in intact cells. It is known that the ability of APT1 to depalmitoylate thioacylated G α_s is regulated by activation of the G protein in that APT1 preferentially depalmitoylates G α_s when the G α subunit is dissociated from its inhibitory interactions with its G $\beta\gamma$ subunits (12). We hypothesized that APT1 may also prefer the activated form of eNOS as a substrate because it is known that eNOS depalmitoylation follows activation of eNOS in cells (11). Because Ca²⁺-CaM binding is thought to induce an activating conformational change in eNOS (reviewed in Ref. 21), Ca²⁺-CaM was included in the *in vitro* depalmitoylation reaction. Fig. 4 demonstrates that the addition of Ca²⁺-CaM potentiates depalmitoylation of eNOS by APT1. Without exogenous APT1, Ca²⁺-CaM alone does not significantly increase depalmitoylation over that seen in buffer alone, suggesting that enhanced depalmitoylation of the activated form of eNOS is not simply due to possible greater accessibility of the thioester bond to nucleophiles in the buffer secondary to the conformational changes that follow Ca²⁺-CaM binding. Consistent with a role for Ca²⁺-CaM in promoting eNOS depalmitoylation is the observation that the addition of EGTA significantly attenuates APT1 acceleration of eNOS depalmitoylation (Fig. 4).

The enhanced APT1 depalmitoylation of eNOS observed when eNOS is activated by Ca²⁺-CaM is specific in that APT1 depalmitoylation of G α_s is not accelerated by the addition of Ca²⁺-CaM (Fig. 5). The inability to potentiate or inhibit APT1 depalmitoylation of G α_s by Ca²⁺-CaM and EGTA, respectively (Fig. 5), and the lack of effect of Ca²⁺-CaM and EGTA on the inability of APT1 to depalmitoylate caveolin (Fig. 6) both support the specificity of activation-dependent APT1 depalmitoylation of its substrates.

The high-energy thioester bond of the palmitate donor palmitoyl-CoA in conjunction with the lack of identifiable palmitoylation consensus sequences and the failure to date to isolate protein palmitoyltransferases raise the possibility that protein palmitoylation may not require a specific enzyme for catalysis. Furthermore, because protein palmitoylation can be potentially regulated in two ways (by a palmitate-adding palmitoyltransferase and/or by a palmitate-removing palmitoylthioesterase), one can envision a dynamic acylation-deacylation cycle carried out by an activation-dependent deacylating palmitoylthioesterase and constitutive nonenzymatic palmitoylation. The possibility of protein autopalmitylation is supported by

data that certain heterotrimeric G protein α subunits (17) and several other proteins (25–30) are spontaneously autopalmitylated at the relevant Cys residues when incubated *in vitro* with palmitoyl-CoA.

Whether APT1 is the only enzyme responsible for regulating the palmitoylation state of eNOS and other palmitoylated proteins will be ultimately studied by genetic disruption of the APT1 gene in an appropriate cell or animal model. Although it is interesting to speculate that the substrates of APT1 may consist of those signaling proteins that are dependent on palmitoylation for dynamic attachment to the membrane, additional palmitoylated peripheral membrane proteins (such as the Src family tyrosine kinases) and integral membrane proteins (such as the β_2 -adrenergic receptor) must be tested as potential substrates before reaching definitive conclusions about the substrate specificity of APT1. Although much remains to be explored, this study provides evidence that eNOS depalmitoylation may be enzymatically regulated in a physiologically relevant manner by the recently identified protein palmitoylthioesterase APT1. We have identified a broadened substrate specificity for APT1 that may provide a general mechanism for the concerted depalmitoylation of structurally dissimilar signaling proteins following their activation by common extracellular signals.

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REFERENCES

1. Michel, T., and Feron, O. (1997) *J. Clin. Invest.* **100**, 2146–2152
2. Robinson, L. J., and Michel, T. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92**, 11776–11780
3. Boutin, J. A. (1997) *Cell. Signal.* **9**, 15–35
4. Dunphy, J. T., and Linder, M. E. (1998) *Biochim. Biophys. Acta* **1436**, 245–261
5. Shenoy-Scaria, A. M., Dietzen, D. J., Kwong, J., Link, D. C., and Lublin, D. M. (1994) *J. Cell Biol.* **126**, 353–363
6. Song, K. S., Sargiacomo, M., Galbiati, F., Parenti, M., and Lisanti, M. P. (1997) *Cell Mol. Biol.* **43**, 293–303
7. Shaul, P. W., Smart, E. J., Robinson, L. J., German, Z., Yuhanna, I. S., Ying, Y., Anderson, R. G. W., and Michel, T. (1996) *J. Biol. Chem.* **271**, 6518–6522
8. Mouillac, B., Caron, M., Bonin, H., Dennis, M., and Bouvier, M. (1992) *J. Biol. Chem.* **267**, 21733–21737
9. Hayashi, M. K., and Haga, T. (1997) *Arch. Biochem. Biophys.* **340**, 376–382
10. Wedegaertner, P. B., and Bourne, H. R. (1994) *Cell* **77**, 1063–1070
11. Robinson, L. J., Busconi, L., and Michel, T. (1995) *J. Biol. Chem.* **270**, 995–998
12. Duncan, J. A., and Gilman, A. G. (1998) *J. Biol. Chem.* **273**, 15830–15837
13. Sugimoto, H., Hayashi, H., and Yamashita, S. (1996) *J. Biol. Chem.* **271**, 7705–7711
14. Lee, E., Linder, M. E., and Gilman, A. G. (1994) *Methods Enzymol.* **237**, 146–164
15. Lamas, S., Marsden, P. A., Li, G. K., Tempst, P., and Michel, T. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89**, 6348–6352
16. Bredt, D. S., and Schmidt, H. H. W. (1996) in *Methods in Nitric Oxide Research* (Feelisch, M., and Stamler, J. S., eds) pp. 249–258, John Wiley & Sons, Inc., New York
17. Duncan, J. A., and Gilman, A. G. (1996) *J. Biol. Chem.* **271**, 23594–23600
18. Busconi, L., and Michel, T. (1993) *J. Biol. Chem.* **268**, 8410–8413
19. Feron, O., Belhassen, L., Kobzick, L., Smith, T. W., Kelly, R. A., and Michel, T. (1996) *J. Biol. Chem.* **271**, 22810–22814
20. Mumby, S. M., and Gilman, A. G. (1991) *Methods Enzymol.* **195**, 215–233
21. Nathan, C., and Xie, Q. (1994) *J. Biol. Chem.* **269**, 13725–13728
22. Michel, J. B., Feron, O., Sacks, D., and Michel, T. (1997) *J. Biol. Chem.* **272**, 15583–15586
23. Couet, J., Sargiacomo, M., and Lisanti, M. P. (1997) *J. Biol. Chem.* **272**, 30429–30438
24. Dietzen, D. J., Hastings, W. R., and Lublin, D. M. (1995) *J. Biol. Chem.* **270**, 6838–6842
25. Bizzozero, O. A., McGarry, J. F., and Lees, M. B. (1987) *J. Biol. Chem.* **262**, 13550–13557
26. O'Brien, P. J., St. Jules, R. S., Reddy, T. S., Bazan, N. G., and Zatz, M. (1987) *J. Biol. Chem.* **262**, 5210–5215
27. Ross, N. W., and Braun, P. E. (1988) *J. Neurosci. Res.* **21**, 35–44
28. Qanbar, R., and Possmayer, F. (1994) *FASEB J.* **8**, A1355 (abstr.)
29. Bharadwaj, M., and Bizzozero, O. A. (1995) *J. Neurochem.* **65**, 1805–1815
30. Bano, M. C., Jackson, C. S., and Magee, A. I. (1998) *Biochem. J.* **330**, 723–731