Published in final edited form as:
Circ Res. 2012 May 11; 110(10): 1336–1344. doi:10.1161/CIRCRESAHA.112.269514.

Endothelial cell palmitoylproteomics identifies novel lipid modified targets and potential substrates for protein acyl transferases

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Abstract

Rationale—Protein S-palmitoylation is the post-translational attachment of a saturated 16-carbon palmitic acid to a cysteine side chain via a thioester bond. Palmitoylation can affect protein localization, trafficking, stability, and function. The extent and roles of palmitoylation in endothelial cell (EC) biology is not well understood, in part due to technological limits on palmitoylprotein detection.

Objective—To develop a method using acyl-biotinyl exchange (ABE) technology coupled with mass spectrometry to globally isolate and identify palmitoylproteins in EC.

Methods and Results—More than 150 putative palmitoyl proteins were identified in EC using ABE and mass spectrometry. Among the novel palmitoylproteins identified is superoxide dismutase 1 (SOD1), an intensively studied enzyme that protects all cells from oxidative damage. Mutation of cysteine 6 prevents palmitoylation, leads to reduction in SOD1 activity *in vivo* and *in vitro*, and inhibits nuclear localization, thereby supporting a functional role for SOD1 palmitoylation. Moreover, we used ABE to search for substrates of particular protein acyl transferases in EC. We found that palmitoylation of the cell adhesion protein PECAM1 is dependent on the protein acyl transferase ZDHHC21. We show that knockdown of ZDHHC21 leads to reduced levels of PECAM1 at the cell surface.

Conclusions—Our data demonstrate the utility of EC palmitoylproteomics to reveal new insights into the role of this important post-translational lipid modification in EC biology.

Keywords

endothelium; palmitoylation; proteomics

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Disclosures: none

Introduction

Protein S-acylation is the attachment of a lipid to a cysteine residue in a protein via a thioester linkage [1]. Protein S-acylation is often called “palmitoylation” since the lipid is commonly (but not exclusively) the saturated 16 carbon palmitate; thus “palmitoylation” will be used in this report to denote protein S-acylation. Palmitoylated proteins typically are membrane-associated due to other lipid modifications (such as myristoylation or prenylation) or additional membrane embedded domains. Palmitoylation can affect the trafficking, localization, stability and function of a protein [2]. Unlike other lipid modifications, palmitoylation is reversible and likely regulated.

Two major developments over the last several years have dramatically changed the study of protein palmitoylation. The first has been the introduction of powerful new methods to detect palmitoylproteins. Classically, palmitoylation was detected primarily by metabolic labeling of proteins with tritiated palmitate followed by fluorography. This approach is time consuming (requiring weeks to months for film exposures), insensitive, and requires advance knowledge of the palmitoylprotein. More recently, palmitoylproteins have been characterized using acyl-biotinyl exchange (ABE) [3–5] or metabolic labeling with palmitate analogs amenable to chemical derivatization with reporter groups, typically using copper-catalyzed cycloaddition (“click”) chemistry [6, 7]. ABE involves first blocking free thiols on isolated proteins, followed by cleaving palmitoyl groups with the neutral base hydroxylamine (HA), and the derivatization of the newly formed thiols using reporter groups such as biotin (Supplemental Fig. 1). ABE and “clickable” palmitate analogs [8, 9] are much more powerful techniques than tritiated palmitate since they are very sensitive, and can be performed relatively rapidly [10]. Furthermore, unlike tritiated palmitate, neither approach necessarily requires advanced knowledge of the palmitoylprotein and both can be used to isolate total palmitoylproteins from cells or tissues. These approaches have been applied to the elucidation of all palmitoylproteins from a variety of sources including yeast [4], and several mammalian cell types [3, 6, 7, 11–14].

The second development has been the discovery of the zinc finger DHHC-domain containing (ZDHHC) family of protein acyl transferases (PAT) [15–17], thus identifying the long sought enzymatic activity catalyzing protein palmitoylation. There are 23 ZDHHC family members in humans characterized by the presence of a highly conserved tetrapeptide repeat (Asp-His-His-Cys, or DHHC in single letter code) that lies within a larger conserved cysteine rich domain. ZDHHC proteins have multiple predicted transmembrane domains, and have been found in multiple membrane compartments including ER, Golgi, and plasma membrane [18]. ZDHHC family members are likely responsible for the palmitoylation of most palmitoylproteins, based on experiments in yeast showing that the palmitoylation of nearly all known palmitoylproteins could be attenuated by the deletion of one or more ZDHHC genes [4]. An increasing array of diseases in mice and humans has been associated with mutations in various ZDHHC family members [19–21]. These data suggest that although there are multiple PAT paralogs, they are likely not functionally redundant, and may serve critical independent functions *in vivo*. Many questions regarding the ZDHHC family of PATs remain unanswered including the nature of their substrate specificity and regulation.

Protein palmitoylation has not been well studied in the context of endothelial cells (EC), the major cell type localized at the interface of blood and tissue representing the largest endocrine organ in the body [22]. Earlier work from our lab has shown that endothelial nitric oxide synthase (eNOS), an enzyme which produces the gaseous messenger nitric oxide is palmitoylated at two cysteines in the amino terminus [23]. Palmitoylation of eNOS is important for trafficking to the plasmalemmal membrane domains, termed caveolae, and for

its cellular activity [24]. eNOS can be palmitoylated by several Golgi-localized ZDHHC enzymes and siRNA-mediated knockdown of one of these (ZDHHC21) leads to mislocalization of eNOS and impaired nitric oxide production [25].

Since the endothelium is critical for many aspects of vascular health and disease, we used ABE to globally isolate palmitoylproteins from EC and to begin to search for novel substrates for ZDHHC proteins that regulate EC function. Here, we report the identification of over 150 palmitoylprotein candidates, many of which have not been reported previously. In particular, we identify superoxide dismutase 1 (SOD1) as an unexpected palmitoylprotein. Mutation of C6 on SOD1 blocks palmitoylation and diminishes enzymatic activity and nuclear localization, thus supporting an important biological role for SOD1 palmitoylation. In addition, using ABE in combination with siRNA-mediated knockdown of two ZDHHC paralogs enriched in EC we show that palmitoylation of the adhesion protein PECAM1 is dependent on ZDHHC21. Knockdown of ZDHHC21 is shown to affect PECAM1 protein levels. Thus, the application of ABE-based palmitoylproteomics expands the scope of palmitoylation in endothelial cells and suggests that ZDHHC enzymes are likely potential targets for vascular diseases.

Methods

Cell culture

EA.hy 926, human umbilical vein endothelial cells (HUVEC), HEK 293T and COS-7 cells were using standard techniques. HEK 293T cells and COS-7 cells were transfected using either Fugene or Lipofectamine 2000 according to manufacturer's instructions.

Acyl-Biotinyl Exchange

ABE was performed based on a published protocol [5] with minor modifications; see online Supplement for additional details. Confluent EA.hy 926 or HUVEC (passage ≤5) cells were lysed in 2ml of lysis buffer (50mM Tris, 5mM EDTA, 150mM NaCl, 1.7% β octyl glucoside or 1% NP-40, 10% glycerol, 5mM $MgCl_2$, 4.5 mg/ml sodium pyrophosphate, 2.1 mg/ml NaF, 0.3mg/ml AEBSF, 1 mM $NaVO_3$, 2 mg/ml complete protease inhibitor tablet (Roche), pH 7.4) per C150 plate. Extracted proteins (2–8 mg) were then precipitated using several volumes of ice cold acetone. Following resuspension, free thiols were then blocked by addition of with 20mM methyl methanethiosulfonate (Fluka). Following removal of excess MMTS via protein precipitation and resuspension, the solution was split in half. Both halves were treated with 1 mM Biotin-HPDP (Thermo Scientific). Simultaneously, one half was treated with HA (NH_2OH), pH 7.4, 1M final concentration, and the other with Tris buffer pH 7.4 as a control. Biotinylated proteins were then captured on streptavidin-conjugated resin (Sigma Aldrich), washed, and eluted with 1% β-mercaptoethanol.

Subsequently the proteins were analyzed by Western blotting and/or by liquid chromatography-MS/MS. Raw LC-MS/MS spectra were assigned to proteins using Mascot software (www.matrixscience.com).

Western Blotting

SDS PAGE, electroblotting, and probing with primary and secondary antibodies were performed using standard techniques.

Immunofluorescence of SOD1

COS cells were grown on glass coverslips and transfected with HA-tagged SOD1. SOD1 was detected with anti-HA antibody (Roche 3F10) and goat anti-rat-488 (Invitrogen) and

visualized using a Leica TCS SP5 Spectral Confocal Microscope. Quantification was performed by a blinded independent judge.

Immunopurification of SOD1

SOD1 WT or C6S, labeled with an HA-affinity tag at the carboxyl terminus, was transfected in HEK-293T cells. SOD1 was immunoprecipitated from cell lysates using sepharose resin conjugated with anti-HA antibody (Sigma) overnight, and then eluted with 100 μg/ml anti-HA peptide (Sigma) in wash buffer.

Tritiated palmitate incorporation

HEK 293 cells were transfected with WT eNOS, eNOS C15/C26S, WT SOD1, or SOD1 C6S and metabolically labeled with ^{3}H palmitate as described [25]. eNOS or SOD1 was immunopurified from lysates of transfected cells, and analyzed by scintillation counting.

Quantitative RT-PCR

RNA isolation, reverse transcription, and quantitative PCR was performed as described [26], except that GAPDH was used for normalization of transcript abundance.

ROS Levels and SOD activity

ROS generation was measured by the oxidation of 2,7-dichlorofluorescin diacetate (DCF; Molecular Probes). Transfected cells were incubated with DCF 5 μmol/L for 30 min 37°C. and subsequently analyzed by flow cytometry.

Activity of purified SOD1 was determined using the SOD assay kit (Dojindo Molecular Technologies, Inc.) as described by the manufacturer. Purified bovine SOD1 (Sigma) was used to determine a standard curve.

siRNA knockdown

Knockdown of ZDHHC3 and ZDHHC21 in EA.hy 926 cells was performed as described [27], except when scaled up to accommodate larger tissue culture plates. Cells were harvested and analyzed 96h post transfection. All-star nonsilencing control siRNA (Qiagen) was used where indicated.

Statistics

The two-tailed Student's t-test was used to compare treated samples with controls. P values of <0.05 were considered to be significant.

Results

Proteomic analysis of palmitoylated proteins in EC

To appreciate the scope and significance of protein palmitoylation in EC, we globally isolated total palmitoylproteins (in 5 different experiments) from EA.hy 926 cells (an immortalized human EC line) using ABE [5] and subsequently identified the proteins by mass spectrometry (MS) (Supplemental Fig. I). EA.hy 926 cells are a stable human EC line that retains many of the characteristics of primary EC including expression of endothelial cell surface proteins such as ICAM, VCAM, PECAM, and Factor VIII related antigen [28, 29], as well as expression of a similar array of miRNA as those found in HUVEC [30]. All experiments were performed in the absence (−) or presence (+) of HA to discriminate between proteins that do not or do have a labile thioester linkage between palmitate and the thiol. Proteins enriched in the absence of HA likely reflect a false positive.

After combining all the data, candidate palmitoylproteins were defined based on the MS data (see Materials and Methods for description of database searching using Mascot Distiller). Palmitoylproteins were defined as those for which: (1) the Mascot protein score was >56 in the +HA samples; and (2) the Mascot protein score was <56 in the −HA samples. In addition, we screened all the final proteins to ensure that none appeared to be amongst the proteins known to be false positives by ABE such as ubiquitin conjugases, enzymes that use lipoic acid or phosphopantetheine cofactors, or enzymes which act upon acyl-CoA substrates [3].

Using these criteria, >150 palmitoylproteins were classified and are shown in Supplemental Table I. Proteins that were identified but are likely to be either contaminants or false positives are shown in Supplemental Table II. A subset of the identified proteins was confirmed by ABE performed with and without HA, followed by semi-quantitative Western blotting (Fig. 1A). The recovery of the protein in the presence but not the absence of HA demonstrates that the protein is palmitoylated. As seen in Fig. 1A , the known palmitoylproteins eNOS, PECAM1, calnexin, Yes, R-Ras, total Ras, and caveolin-1 and the novel palmitoylprotein, superoxide dismutase (SOD1) are enriched in the presence of HA treatment. Hsp90 and c-Src, which are not palmitoylated, were used as negative controls. Similary, ABE followed by Western blotting confirmed the presence of several palmitoyl proteins, including PECAM1 and SOD1, in primary cultures of HUVEC (Fig. 1B).

Further analysis of the identified proteins was carried out by ascertaining whether the protein was previously identified as palmitoylated by (1) searching published palmitoylproteomes identified via MS in other human cell types [6, 11–13, 31]; (2) by searching for the keyword “palmitate” in human protein entries in the Uniprot database (www.uniprot.org); and (3) performing semiautomated batch Pubmed searches (using the website pmid.us) for every identified palmitoylprotein in conjunction with the search terms “palmitoylation” or “palmitate” or “palmitoyl transferase”. Of all proteins, nearly one third (43 total) were not previously reported. Below we show two examples of advances that have come out of applying ABE and MS technology to the study of EC: (1) the identification of novel, unanticipated palmitoylproteins such as SOD1, and (2) the use of ABE to associate activity of individual PAT paralogs to substrate palmitoylproteins.

Identification of SOD1 as a novel palmitoylprotein

One protein with strong MS evidence supporting its palmitoylation but which has not been described previously is SOD1. SOD1 has been intensively studied and is an important enzyme that protects cells from oxidative damage by converting superoxide anion into hydrogen peroxide. In addition, mutations in SOD1 are associated with the familial variant of amyotrophic lateral sclerosis (fALS), also known as Lou Gehrig’s disease. Examination of the MS data revealed that 8 different peptides mapping to SOD1 were detected with high Mascot scores (and expectation values <0.05) in the +HA samples, but not in the −HA treated samples (Fig. 1C, D). This was confirmed by Western blotting for SOD1 following ABE (Fig. 1A, 1B). Mutagenesis of C6 to serine (S) in SOD1 led to a loss of palmitoylation when expressed in HEK-293T cells (Fig. 1E) as determined by ABE and Western blotting. In addition, the C6S mutant SOD1 displayed reduced incorporation of tritiated palmitate (Fig. 1F), demonstrating that C6 is the likely site of palmitoylation in SOD1.

Next, we explored two possible functional consequences of S-acylation of SOD1. Since palmitoylation affects the subcellular localization of many proteins, we examined the distribution of WT and C6S SOD1 in transfected cells. SOD1 is mainly soluble and localizes in the cytosol as well as in peroxisomes, mitochondria, and nuclei [32]. Expression of WT SOD1 in COS cells resulted in predominate cytoplasmic and nuclear localization (co-labeled with the nuclear marker DAPI) in approximately 70% of the cells examined, whereas C6S

SOD1 was frequently excluded from the nucleus, (Fig. 2A and quantified in Fig. 2B). These results suggest that C6 is involved in the nuclear targeting of SOD1.

We also examined whether palmitoylation affected the enzymatic activity of SOD1. Long chain acylation has previously been reported to alter activity of certain mitochondrial enzymes [33]. Transfection of WT, but not C6S SOD1, into HEK293T cells reduced the levels of reactive oxygen species (ROS) levels as measured by FACS analysis of cells treated with the ROS-sensitive dye DCF (Molecular Probes) (Fig. 2C, 2D). Expression levels of WT and C6S were similar as judged by Western blotting (Fig. 2E), suggesting that the activity of the mutant protein is reduced relative to WT. In order to assess whether this reduction was due to changes in the specific activity of SOD1, we purified WT and C6S HA-tagged SOD1 proteins from HEK cells to near homogeneity using anti-HA resin (Fig. 2F) and assayed enzymatic activity directly. C6S SOD1 displayed a ~30% reduction in specific activity relative to the WT enzyme (Fig. 2G). In sum, these data suggest that palmitoylation of SOD1 at C6 is important for the ability of SOD1 to localize to the nucleus and to optimally scavenge superoxide. It is not clear whether these two phenomena are linked or are unrelated.

Utilization of ABE to assign ZDHHCs and substrates in EC: ZDHHC21 is necessary for optimal palmitoylation of PECAM1

The discovery of the large family of ZDHHC PATs has raised the issue of which paralogs are responsible for palmitoylating which substrate proteins. Previously, we demonstrated that ZDHHC3 and ZDHHC21 are expressed in EC, that both can palmitoylate eNOS when co-expressed in HEK cells, and that siRNA knockdown of ZDHHC21, but not ZDHHC3, in EC impairs eNOS palmitoylation and activity [25]. We sought to discover new substrates for ZDHHC3 and -21 by using siRNA to knockdown both of these PATs (Fig. 3A). In an initial experiment, EC were transfected with control siRNA and siRNA targeting both ZDHHC3 and -21 and the level of palmitoylation after ABE determined by semi-quantitative Western blotting of a group of proteins identified in the EC palmitoylome. As seen in Fig. 3B, the knockdown of ZDHHC3 and -21 markedly reduced the palmitoylation of PECAM1 (aka CD31) and to lesser extent, eNOS, calnexin and syntaxin-6. The loss of these ZDHHCs did not influence the palmitoylation of MCAM/MUC18, Yes, $G\alpha_i$, R-Ras, total Ras or caveolin-1, thus demonstrating the specificity of the effect. PECAM1 is a type I transmembrane protein that is expressed in EC and several circulating blood cells. In the endothelium, it serves several important roles in cell migration [34, 35], transendothelial migration of leukocytes [36], flow sensing [37], and angiogenesis [38, 39] among other functions. Palmitoylation of PECAM1 on C595 in platelets was recently described [40] where it was found to be important for localization of PECAM1 to lipid rafts and for mediating inhibition of stimulated apoptosis.

To determine which PAT was necessary for PECAM1 palmitoylation, each gene was knocked down alone in EC followed by ABE and Western blotting for PECAM1. As seen in Fig. 3C, palmitoylation of PECAM1 in EC was primarily dependent on ZDHHC21, not ZDHHC3. In order to assess the functional consequences of ZDHHC21 dependent PECAM1 palmitoylation in EC, we examined whether knockdown of ZDHHC21 affected steady state levels of endogenous PECAM1. The trafficking, breakdown, and cell surface localization of several other integral membrane proteins have been shown to be affected by palmitoylation [41–44]. Knockdown of ZDHHC21, but not ZDHHC3, reduced levels of PECAM1 in cell lysates by ~50% (Fig. 3C and quantified in 3D). Similarly, FACS analysis of cell surface PECAM1 in non-permeabilized EC following knockdown of ZDHHC21 revealed a marked reduction in PECAM1 levels on the cell surface (Fig. 3E). Thus, ZDHHC21 regulates the levels of PECAM1 in EC.

Discussion

Palmitoylation has long been recognized as an important post-translational modification that can affect protein localization, stability, and function. Using ABE followed by an unbiased proteomic approach, we identify >150 candidate palmitoyl proteins in EC. Confidence in the veracity of the identified candidate palmitoylproteins is inherent in the fact that more than half of the identified proteins have previously been reported either by direct biochemical analysis or in other global palmitoylproteomic studies of human cells. In addition, several proteins identified here were confirmed by ABE followed by Western blotting (Fig. 1A, B) and mutagenesis studies (e.g., Fig. 1D). Thus, our results provide the first global approach in EC and are an important step forward in the understanding of the role of palmitoylation in this specialized cell type that regulates aspects of cardiovascular disease, cancer and diabetes.

One interesting novel palmitoylprotein characterized in the present study is SOD1. SOD1 expressed in EC is of particular importance in the pathophysiology of variety of diseases associated with elevated oxidative stress such as atherosclerosis, diabetes and fALS. SOD1 has been extensively characterized in biochemical and biophysical experiments, including the determination of several crystal structures [45]. Thus, our detection of SOD1 as a palmitoylprotein was not anticipated. The failure to detect palmitoylation in previous studies may be due to many issues such as the presence of reductant in most purification schemes (i.e. the thioester would be reduced), and the usage of inappropriate expression and purification strategies combined with insensitive or unsuitable analytical techniques. Interestingly C6 appears to be buried in the crystal structure of the mature form of SOD1 [45]. Thus, we suspect that acylation may occur initially in the apoprotein of SOD1, where NMR structural data reveal that C6 is solvent exposed and thus accessible[46]. Significant quantities of apo-SOD1 are thought to exist in the cell at steady state [47]. Alternatively, it is conceivable that the acyl group is cleaved during maturation of SOD1.

The cysteines of SOD1 have been extensively studied previously by several laboratories in a number of different contexts. The C6F mutation has been found to be among the many mutations that cause fALS and destabilize the protein [48]. The more conservative C6A mutation was found to slightly enhance rather than inhibit stability [49]. Of note, the introduction of the C6S mutation in the context of fALS-associated mutations inhibits protein aggregation in experimental studies [50], possibly by blocking intermolecular disulfide formation involving C6 (and other cysteines) [51–53], thus, palmitoylation of C6 may serve as an endogenous mechanism to mitigate the reactivity of C6, especially in the aggregation-prone apoprotein associated with causal fALS mutations.

Confocal microscopy studies of the SOD1 C6S mutant suggest that nuclear localization is impaired (Fig. 2A, 2B). Though nuclear localization of SOD1 has been previously recognized [32], the mechanism by which SOD1 is imported into the nucleus is not known. In general, palmitoylation of nuclear proteins has not been well described; but, recent data suggests that palmitoylation may be important for the nuclear localization and transcriptional activity of the transcription factor HMGCS2 [54] and for the nuclear histone H3 [31]. The relationship between the decrease in enzyme activity and the alteration of nuclear localization observed in the SOD1 C6S mutant is not clear and the subject of further exploration. In addition, it is appreciated that SOD1 can exist in the nucleus in many cells, but the functional importance of SOD1 subcellular localization and its role in EC biology are unknown.

Another salient finding in this study is the identification of relationships between individual ZDHHC paralogs and potential substrates in EC. To date, most success in identifying such

relationships has come from studies designed to define the set of ZDHHC paralogs which can augment palmitoylation of a particular substrate protein. These experiments usually involve co-transfection of a substrate protein together with each of the known ZDHHC proteins in HEK 293 cells, followed by determination of the level of palmitoylation using radioactive palmitate [55]. However, the inverse experiment, i.e., determination of all the substrates of a given ZDHHC paralog, is more relevant to understanding the biological role of ZDHHC enzymes and to dissecting the molecular mechanisms of the growing array of *in vivo* phenotypes that are caused by defects in particular ZDHHC family members.

To this end, we performed ABE after siRNA-mediated knockdown of ZDHHC21 and -3, two paralogs found in EC that palmitoylate eNOS[25] and have identified PECAM1 as a palmitoylprotein sensitive to the loss of ZDHHC21. This knockdown/ABE approach has the advantage of not being subject to artifacts related to overexpression of proteins in a heterologous cell type. Similar approaches have been used in yeast using strains deficient for various PATs [4], in HeLa cells, where an interaction between CKAP4/p63 and ZDHHC2 was identified [56], and most recently, in mouse neural stem cells derived from a mouse with a hypomorphic ZDHHC5 allele [57]. Of note, however, neither the knockdown nor the overexpression strategies proves a direct enzyme-substrate relationship. Additional studies, perhaps using purified proteins in a reconstitution system, will be required to fully understand the nature of the interaction between a ZDHHC protein and candidate substrate palmitoylprotein.

Several other potential substrates have been reported for ZDHHC21 in addition to PECAM1, including eNOS [27], Fyn [19], and Lck [58]. There are known connections amongst these proteins: eNOS and PECAM1 may interact directly to regulate NO production in response to flow [59, 60], and Fyn [61] and Lck [62, 63] are reported to phosphorylate PECAM1. These data raise the intriguing possibility that ZDHHC21 may be a global integrator of interactions amongst related signaling molecules in EC.

Functionally, we found that knockdown of ZDHHC21 led to reduced levels of PECAM1, especially at the plasma membrane. Palmitoylation of plasma membrane proteins may affect cell surface expression in a variety of ways, including by altering trafficking, increasing internalization, and augmenting degradation [64]. Of note, a recent quantitative proteomic analysis [57] comparing WT and ZDHHC5 hypomorphic neural stem cells revealed that all of the 20 proteins in which palmitoylation was most reduced in the mutant cells also displayed reductions in total protein levels (when data were available). Thus, decreased in protein abundance in the setting of reduced palmitoylation, as was observed for PECAM1 (Fig. 3C, D), may be a common occurrence.

In sum, utilizing ABE technology and MS in EC for the first time, we have discovered several new palmitoylproteins and have begun to assign relationships between ZDHHC paralogs and palmitoylproteins. Specifically, we show that SOD1 is palmitoylated, and that palmitoylation may regulate its nuclear localization and activity. In addition, using siRNA knockdown of two ZDHHC enzymes, we have shown that ZDHHC21 is required for the palmitoylation of the junctional adhesion molecule PECAM1 and regulates its residence and/or stability in the plasma membrane. Future studies using ABE technology will further investigate how growth factors or hemodynamic forces alter the composition of the palmitoylome delineating ZDHHC-substrate pairs as potential targets to regulate EC functions. These experiments will help define the role of S-palmitoylation in EC biology.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

none

Source of Funding: This work was supported by grants R01 HL64793, R01 HL61371, R01 HL081190, HL096670, P01 HL70295 and Contract No. N01-HV-28186 (NHLBI-Yale Proteomics Contract) from the National Institutes of Health to W.C. Sessa; a National Kidney Foundation Post-doctoral fellowship, the 5T32DK007276-33 Institutional Research Training Grant, and K08 HL103831 Career Development Award to E.P. Marin.

Non-standard abbreviations and acronyms

ABE	acyl-biotinyl exchange
EC	endothelial cell
eNOS	endothelial nitric oxide synthase
fALS	familial amyotrophic lateral sclerosis
HUVEC	human umbilical vein endothelial cells
HA	hydroxylamine
MS	mass spectrometry
PAT	protein acyl transferase
PECAM1	platelet/endothelial cell adhesion molecule 1
ZDHHC	zinc finger DHHC domain containing protein

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Novelty and Significance

What is known?

- Palmitoylation is the reversible post-translational modification of a protein by the attachment of a lipid to a cysteine side-chain via a thioester linkage. Palmitoylation can influence protein localization, activity or stability.
- Palmitoylation is catalyzed by the large family of ZDHHC protein acyl transferases.
- Palmitoylation affects the function of several proteins critical to endothelial cell biology such as eNOS and caveolin-1, but many palmitoylated proteins in endothelial cells have not been identified due to technical difficulties.

What new information does this article contribute?

- Acyl biotinyl exchange, a new method for globally isolating palmitoylated proteins, was applied to endothelial cells and over 150 palmitoyl proteins were isolated and identified by mass spectrometry.
- Superoxide dismutase 1, a protein important in protecting endothelial cells from the toxicity of superoxide radical, was found to be palmitoylated.
- The protein acyl transferase ZDHHC21 was found to be associated with the palmitoylation of the cell adhesion molecules PECAM1 in endothelial cells

Palmitoylation is known to be important for the function of several endothelial cell proteins, but an understanding of the full scope of palmitoylation in endothelial biology has been limited by technical difficulties in identifying which proteins are palmitoylated. Therefore, for the first time using a recently-developed unbiased approach, called acyl biotinyl exchange, we analyzed palmitoylproteins in endothelial cells. We identified >150 candidate palmitoylproteins, many of which are novel. We show that superoxide dismutase 1 (SOD1), is palmitoylated and that this modification alters its subcellular localization and its catalytic activity. Additionally, we show that acyl biotinyl exchange can be used to approach an important question regarding identification of specific substrates for individual members of the large family of ZDHHC protein acyl transferases. In particular, we identify a novel relationship between ZDHHC21 and palmitoylation of PECAM1, an important endothelial cell adhesion protein. Collectively, these results expand our understanding of the scope of palmitoylation in endothelial cells, and identify strategies for further unraveling the role of this modification and the related ZDHHC protein acyl transferase enzymes in endothelial cells.

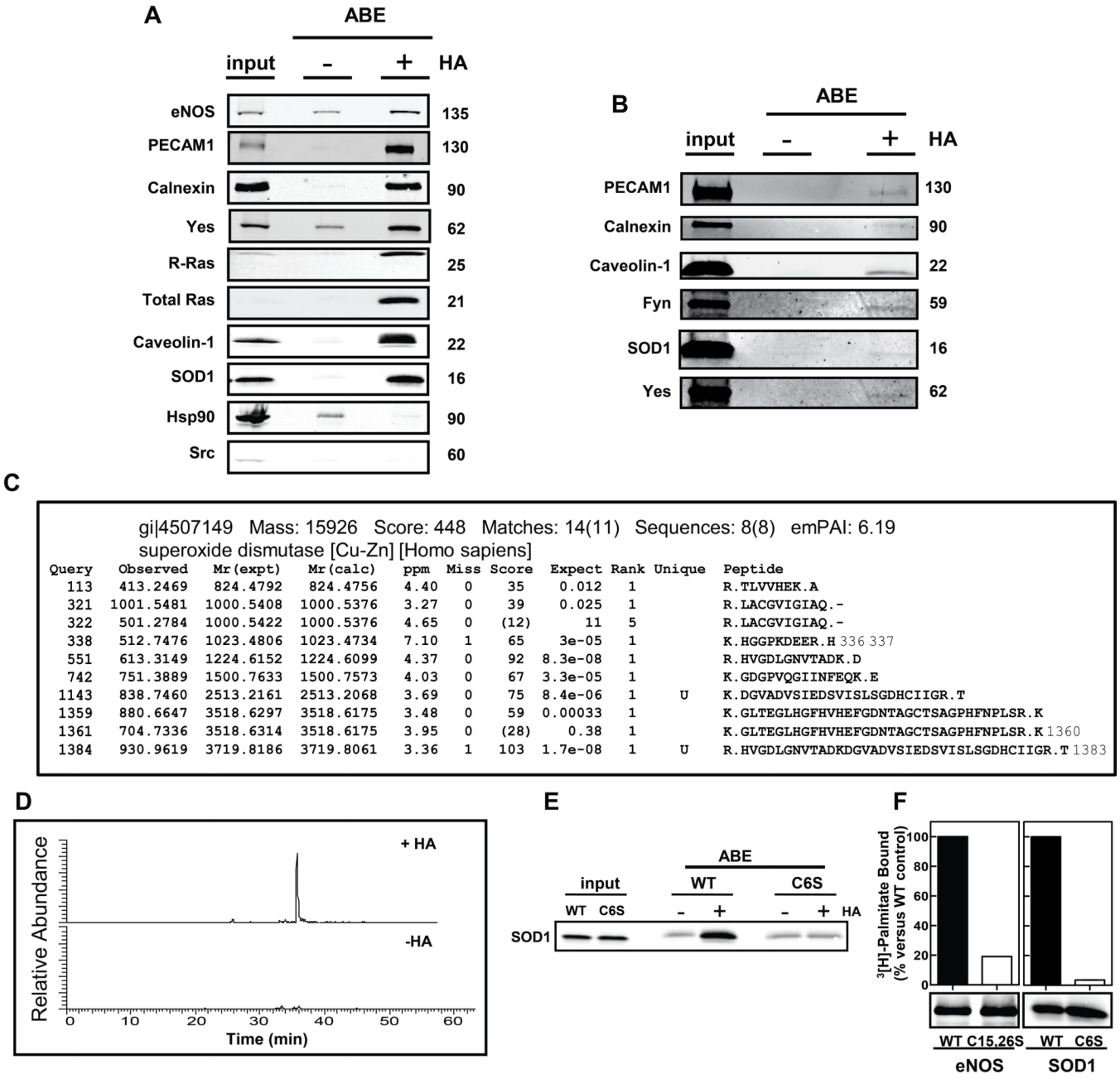

Figure 1. SOD1 is a novel palmitoylprotein

A. A subset of proteins identified by ABE and MS in EA.hy 926 cells was confirmed by ABE and Western blotting. Palmitoylated protein should only be present in samples treated with HA during purification. SOD1 is confirmed as a palmitoylated protein in this analysis. Src and Hsp90 are shown as examples of proteins that do not appear to be palmitoylated. Apparent molecular weights are shown to the right of the blots. B. ABE was also performed in primary HUVEC, where the presence of several of the identified palmitoyl proteins, including PECAM1 and SOD1 is confirmed by Western blotting. C. MS data showing the identification of 8 distinct peptides mapping to SOD1. D. For one of the peptides (with m/z 751.3889), the raw extraction ion spectrogram is shown to demonstrate the presence of the peptide only in samples treated with HA. E, WT SOD1, heterologously expressed in HEK

293T cells, is palmitoylated but the C6S mutant is not based on ABE followed by Western blot for SOD1. F, Incorporation of tritiated palmitate as measured by scintillation counting of heterologously expressed proteins following immunopurification. The SOD1 C6S mutant blocks incorporation of tritiated palmitate relative to WT. As a control, WT and palmitoylation deficient C15S/C26S mutant of eNOS is shown. Similar amounts of protein recovered by immunopurification are confirmed by Western blotting. The data are the mean of two independent experiments performed in triplicate.

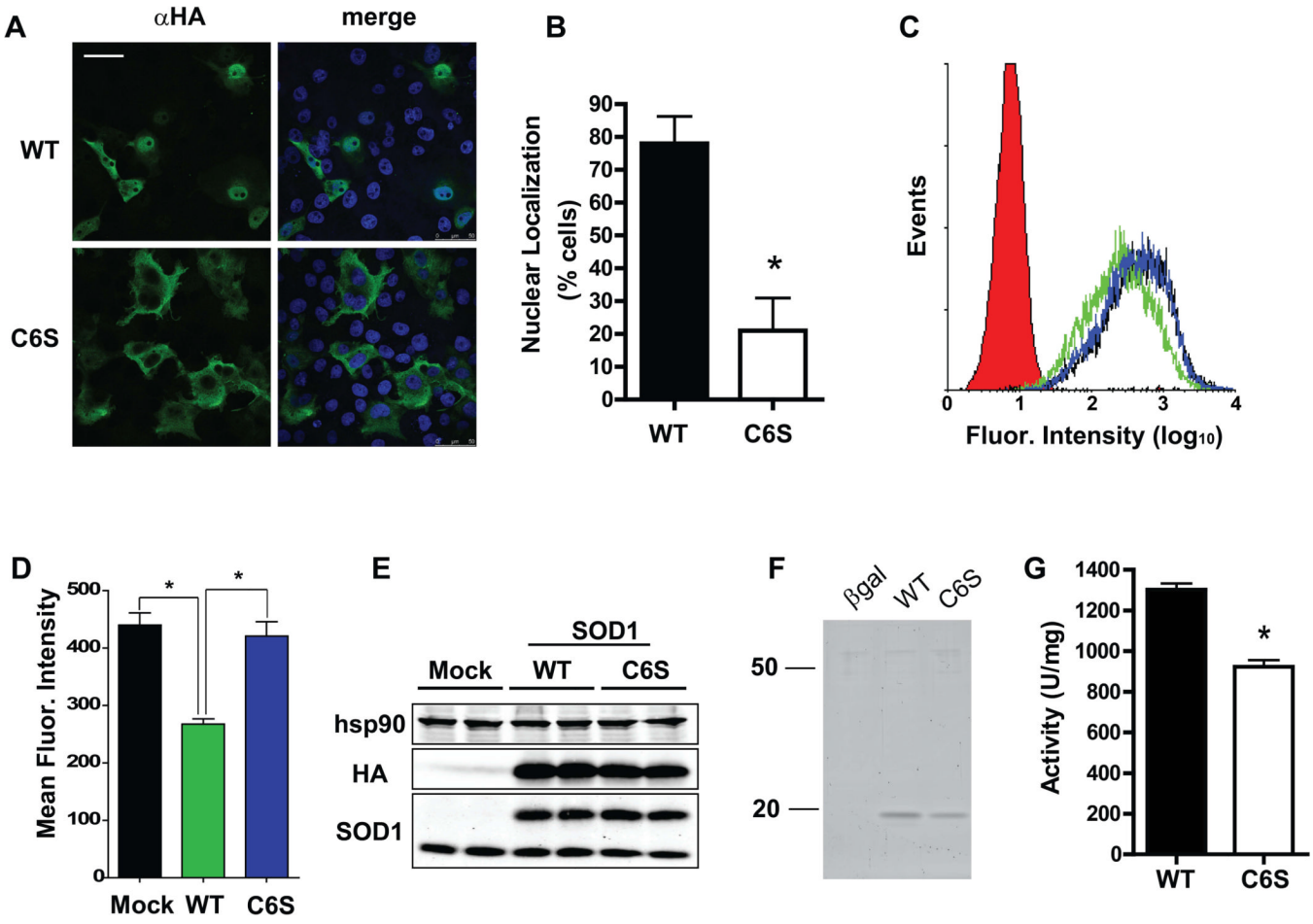

Figure 2. The SOD1 C6S mutant displays decreased nuclear localization and less activity compared with WT

A, SOD1 C6S is present at lower levels in the nucleus relative to SOD1 WT. COS cells were transfected with the indicated HA-tagged construct, stained with anti-HA antibody and a fluorescently tagged secondary antibody, and analyzed by confocal microscopy. Representative images are shown. Scale bar = 50 μm. B, Quantification of nuclear localization was performed by a blinded independent observer who scored nuclei for the presence or absence of significant HA staining. n= 3 independent experiments; in total, 66 WT and 72 C6S cells were analyzed. *, $p<0.05$ C, Transfection of HA-tagged WT but not C6S SOD1 led to a reduction in reactive oxygen species as indicated by FACS of HEK 293T cells loaded with the dye DCF. D, Quantification of the mean fluorescence intensity of FACS data from 3 independent experiments performed in triplicate. *, $p<0.05$. E, Western blot shows similar levels of expression of endogenous and transfected SOD1 in lysates from the transfected HEK cells. Hsp90 is shown as a loading control. F, Purification of recombinant SOD1. Heterologously expressed HA-tagged SOD1 WT and C6S mutants were purified using resin conjugated with HA-antibody and eluted using HA epitope peptide. SDS-PAGE stained with sypro-ruby confirms high degree of purity. G, The specific activity of recombinant C6S SOD1 is lower than WT SOD1. Activity was measured in triplicate by assessment of inhibition of superoxide production generated by xanthine oxidase/xanthine. Similar results were obtained from two individual protein preparations.

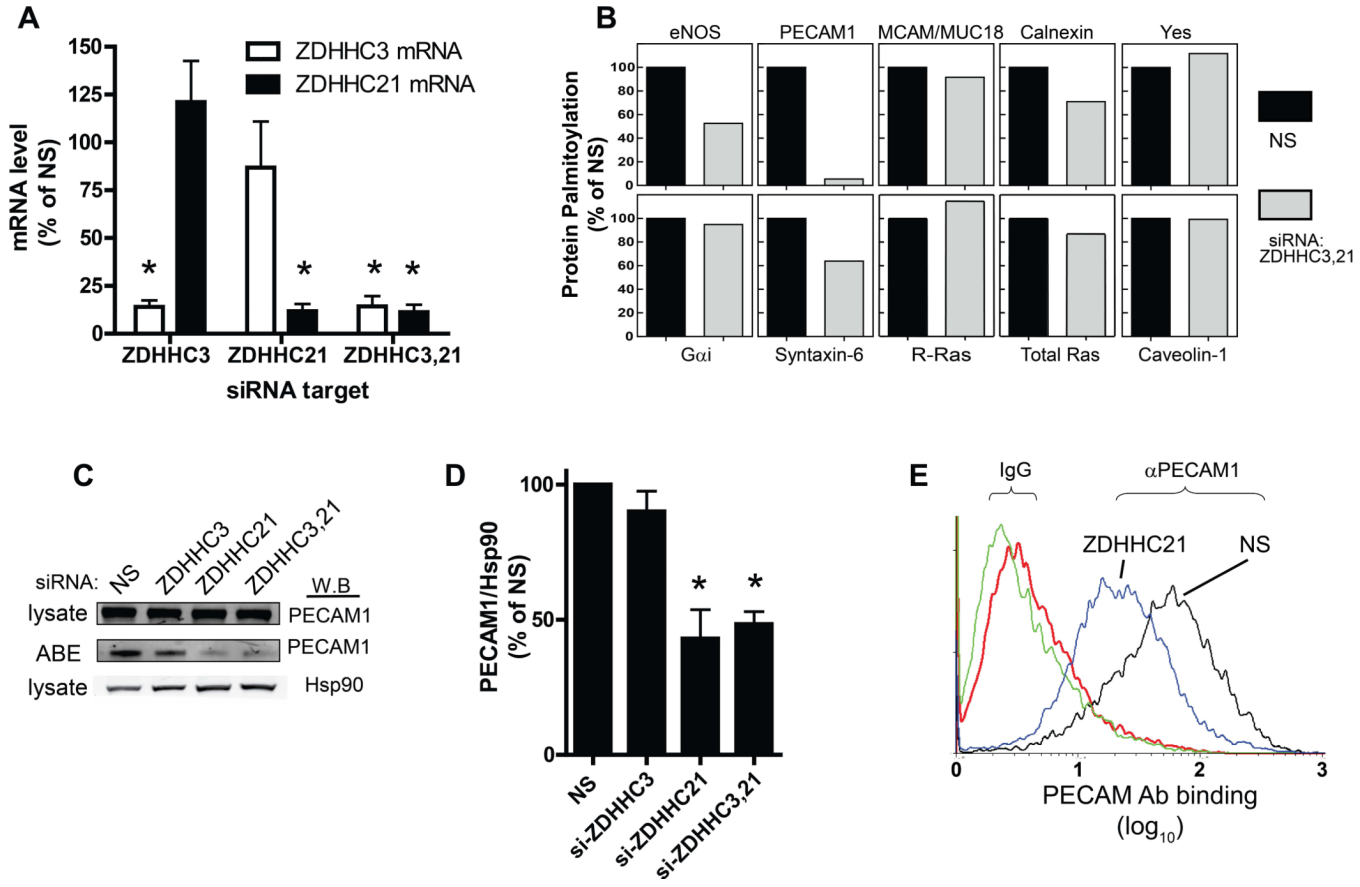

Figure 3. PECAM1 palmitoylation and cell surface expression is dependent on ZDHHC21 in EC

A, Validation of siRNA mediated knockdown of ZDHHC3 and ZDHHC21. EA.hy 926 cells were treated with siRNA oligonucleotides targeted against ZDHHC3, ZDHHC21, or nonsilencing (NS) control. Abundance of indicated mRNA was assessed by qPCR. n=4 independent experiments; *p<0.05 compared with NS. B, Screen for substrates of ZDHHC3 and ZDHHC21. EA.hy 926 were treated with either NS control siRNA or siRNA targeted to ZDHHC3 and -21. ABE was performed, and the degree of palmitoylation for 10 different proteins in the EC palmitoylproteome was assessed by Western blotting and quantitated by densitometry. PECAM1 palmitoylation was found to be particularly sensitive to knockdown of ZDHHC3 and -21. C, PECAM1 palmitoylation is sensitive to ZDHHC21 but not ZHDDC3 knockdown as judged by ABE and Western blotting. Results are typical of three (in the case of the double knockdown) or two independent experiments (in the case of the single knockdowns). Since knockdown of ZDHHC21 can decrease total PECAM1 in the lysates (see below and text), the input of PECAM1 in this experiment was normalized by using increased amounts of total lysate for samples in which ZDHHC21 was knocked down as shown by blot of Hsp90. D, Densitometry on Western blots revealed that PECAM1 levels in lysates (normalized to Hsp90) are reduced by ~50% following 96h of treatment with siRNA targeted to ZDHHC21 but not ZDHHC3. *, p<0.05 relative to NS control, n=3 independent experiments. E, Assessment of PECAM1 cell surface expression by FACS. Treatment with siRNA targeted to ZDHHC21 led to a ~60% reduction in mean fluorescence intensity as compared with cells treated with NS control siRNA. Results are typical of three independent experiments.