Identification of CKAP4/p63 as a Major Substrate of the Palmitoyl Aroyltransferase DHHC2, a Putative Tumor Suppressor, Using a Novel Proteomics Method*

Jun Zhang‡, Sonia L. Planey‡, Carolina Ceballos‡, Stanley M. Stevens, Jr.§, Susan K. Keay¶, and David A. Zacharias‡

Protein palmitoylation is the post-translational addition of the 16-carbon fatty acid palmitate to specific cysteine residues by a labile thioester linkage. Palmitoylation is mediated by a family of at least 23 palmitoyl acyltransferases (PATs) characterized by an Asp-His-His-Cys (DHHC) motif. Many palmitoylated proteins have been identified, but PAT-substrate relationships are mostly unknown. Here we present a method called palmitoyl-cysteine isolation capture and analysis (PICA) to identify PAT-substrate relationships in a living vertebrate system and demonstrate its effectiveness by identifying CKAP4/p63 as a substrate of DHHC2, a putative tumor suppressor. Molecular & Cellular Proteomics 7: 1378–1388, 2008.

The recent discovery of at least 23 enzymes that mediate palmitoylation (palmitoyl acyltransferases or PATs) suggests that regulation of palmitoylation is far more complex than previously assumed. Defining PAT-substrate specificities in living vertebrate systems is critical for understanding the complexity of regulated palmitoylation and the potential consequences of malfunctioning PATs on downstream signaling events. To date, five PAT genes have been implicated in human diseases ranging from schizophrenia to cancer and Huntington disease, so defining the substrates of individual PATs will provide important clues linking these known mutations to an associated disease. One reason why our understanding of palmitoylation has not kept pace with that of other post-translational modifications has been the lack of rapid and sensitive methods to study this phenomenon. Recent advances in the field have provided tools that can identify palmitoylated proteins and match known palmitoyl proteins to PATs already suspected of mediating their palmitoylation (1–3). But until now, there has been no method to identify substrates of specific PATs in a living vertebrate system without prior knowledge of a possible relationship between PAT and substrate. Palmitoyl-cysteine isolation capture and analysis (PICA) solves this problem. In this study, we used PICA and HeLa (human cervical carcinoma cells) to identify CKAP4/p63 as a physiologically important substrate of DHHC2, a PAT that has been linked to the metastatic potential of liver and colon cancer (4).

The ability of PICA to identify PAT substrates is based on the principle that it quantifies the differential frequency of palmitoylation of individual proteins in control conditions versus conditions in which the function of a single PAT is reduced by siRNA-mediated gene knockdown (Fig. 1). The process to identify substrates of DHHC2 consisted of four basic steps. In the first part we generated two distinct pools of palmitoylated proteins in HeLa cells: one from untreated, control HeLa cells and the other from HeLa cells in which the activity of one PAT, DHHC2, was reduced. These two distinct pools of palmitoylated proteins were then captured, and the differences in the degree of palmitoylation of individual proteins among the two pools were identified. To do this, first we reduced the expression of ZDHHC2 mRNA (and consequently the function of the encoded enzyme, DHHC2) in HeLa cells using siRNA-mediated gene knockdown, resulting in a reduced level of palmitoylation of DHHC2 substrates. Total protein from knockdown and control cells was prepared by first blocking all free (including non-palmitoylated) thiols with methyl methanethiosulfonate (MMTS). This was followed by selective exposure of all

From the †The Whitney Laboratory, Department of Neuroscience, University of Florida, St. Augustine, Florida 32080, §Proteomics Core Facility, Interdisciplinary Center for Biotechnology Research, University of Florida, Gainesville, Florida, 32610, and ¶Veterans Affairs Medical Center, University of Maryland, Baltimore, Maryland 21201

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1 The abbreviations used are: PAT, palmitoyl acyltransferase; DHHC, Asp-His-His-Cys; PICA, palmitoyl-cysteine isolation capture and analysis; GH, Gap43-YFP-His6; YFP, yellow fluorescent protein; siRNA, small interfering RNA; MMTS, methyl methanethiosulfonate; H, heavy; L, light; DMEM, Dulbecco’s modified Eagle’s medium; MDCK, Madin–Darby canine kidney; β-ME, β-mercaptoethanol; TRITC, tetramethylrhodamine isothiocyanate; GFP, green fluorescent protein; 2BP, 2-bromopalmitate; PM, plasma membrane; ER, endoplasmic reticulum; APF, antiproliferative factor.

2 J. Zhang and D. A. Zacharias, poster presented at the Keystone Symposium on Lipid Rafts and Cell Function, Steamboat Springs, Colorado (March 23–28, 2006).
Palmitoyl Acyltransferase Substrate Identification

**EXPERIMENTAL PROCEDURES**

**ZDHHC Knockdown by siRNA Transfection of HeLa Cells**—One day prior to transfection, HeLa cells (ATCC number CCL-2) were plated in 10 ml of antibiotic-free DMEM supplemented with 10% fetal bovine serum (per 10-cm plate) so they were at 30–50% confluence. For each transfection, 50 pmol of ZDHHC2 siRNA (20 pmol/μl final concentration) was diluted in 833 μl of serum-containing DMEM, mixed gently, and incubated for 5 min at room temperature with 20 μl of 2 μM transfection reagent. After transfection, cells were incubated for 48 h at 37°C in a humidified 5% CO2 incubator. 

Palmitoyl-cysteines by cleavage of the palmitoyl-cysteine thioester bond with hydroxylamine at neutral pH thereby generating a unique population of cysteines that was formerly palmitoylated. Second, we selectively and differentially labeled the exposed, formerly palmitoylated cysteines from knockdown and control cells with biotinylated, thiol-reactive heavy (H) and light (L) ICAT reagents, respectively. Third, we combined equal quantities of the ICAT-labeled protein from ZDHHC2 knockdown and control cells and digested the mixture with trypsin. The resulting H and L ICAT-labeled tryptic peptides were captured and purified on an avidin affinity column. In the final step, we used mass spectrometry to identify both substrate peptides and the cysteine within the peptide/protein that was palmitoylated. Identification of a DHHC2 substrate was possible because of differential ICAT labeling and subsequent capture of peptides isolated from ZDHHC2 knockdown and control cells; the capture rate for hypopalmitoylated substrate peptides of DHHC2 is reduced. We defined a substrate of DHHC2 as a peptide for which the H/L ICAT ratio was consistently reduced (statistically similar) over four complete, independent PICA runs. We confirmed that CKAP4/p63 is a substrate of DHHC2 in subsequent experiments by showing that co-overexpression of ZDHHC2 and CKAP4/p63 resulted in an increased level of [3H]palmitate labeling of CKAP4/p63 and conversely that there was a decrease in the palmitoylation of immunoprecipitated, endogenous CKAP4/p63 in response to siRNA-mediated knockdown of ZDHHC2 expression. Here we describe the development and validation of PICA and demonstrate the effectiveness of the method by identifying CKAP4/p63 as a principle substrate of DHHC2 in HeLa cells.

Fig. 1. PICA: determining PAT-substrate specificity by differential labeling of palmitoylated proteins with ICAT. A, in one set of cultures, ZDHHC2 expression is knocked down by transfecting HeLa cells with ZDHHC2-specific siRNA (Dharmacon). Proteins are extracted from experimental and control cells and treated with the thiol-specific blocking reagent MMTS. This step chemically modifies or protects all thiols (“X” on the proteins P1–P3) that are free at physiological pH and leaves the palmitoylated cysteines (“P”) undisturbed as depicted on P1–P3. B, following the protection or blocking of free thiols, palmitates are removed by selective cleavage of the thioester bond with hydroxylamine at pH 7.4, which generates a distinctive set of free, formerly palmitoylated, reactive thiols (“C”) that can be selectively labeled with ICAT reagents. The iodoacetamide moiety at one end of the ICAT reagent reacts with the thiol side chain of cysteines; on the other end, biotin provides a mechanism for affinity purification of thiol-captured peptides on an avidin column. C, proteins from knockdown and control conditions are mixed in equal amounts and digested in-gel with trypsin. ICAT-labeled peptides are enriched by avidin affinity purification and analyzed by LC-MS/MS. A pair of ICAT-labeled peptides is chemically identical and is easily visualized because they essentially coelute. The different isotopic composition of the H and L ICATs provides a 9-Da difference in mass that is easily measured by mass spectrometry. Even if equal amounts of a single protein exist in two different samples, the quantity of protein that is captured depends directly on its degree of palmitoylation: if all of a single protein is palmitoylated under one condition, then all of it will be captured; if only half of this protein is palmitoylated under another condition, then the capture rate of that protein will be half as much, relative to control, making it appear half as abundant. Peptide abundance values derived from the areas of commonly shared isotopic peaks were calculated by Analyst/BioAnalyst (Applied Biosystems). Proteins for which there has been no corresponding to the captured peptides after searching the tandem MS data against a protein sequence database.
temperature. 50 μl of Oligofectamine reagent (Invitrogen) were added to 200 μl of serum-free DMEM, mixed gently, and incubated for 5 min at room temperature. The diluted siRNA and Oligofectamine samples were then combined (total volume, 1033 μl, mixed gently, and incubated for 20 min at room temperature to allow siRNA-Oligofectamine complex formation. Complexes were subsequently diluted in 9 ml of antibiotic-free, serum-containing DMEM and added to the HeLa cells. The cells were incubated at 37 °C in a CO2 incubator for 48–72 h until they reached confluency and were ready to assay for gene knockdown.

**Real Time PCR Quantitation of ZDHHC2 mRNA Knockdown in HeLa Cells**—Total RNA was isolated from untreated and siRNA-treated HeLa cells using the RNeasy quick kit and quantitative reverse transcription–PCR. First strand cDNA synthesis was performed using 5 μg of total RNA and Superscript III (Invitrogen) according to the manufacturer’s protocol. The cells were incubated at 37 °C in a CO2 incubator for 48–72 h until they reached confluency and were ready to assay for gene knockdown.

**Validation of PICA Using Heterobifunctional, Thiol-reactive Linkers**—Thiol-reactive affinity columns were prepared according to the manufacturer’s protocol. The affinity matrix consisting of immobilized diaminodipropylamine gel linked to sulfo-LC-SPDP (N-succinimidyl 3-(2-pyridyldithio)-propionate) selectively captures proteins with free, reactive thiols (formerly palmitoylated cysteines) allowing their purification. The columns were rinsed with 10 gel bed volumes of 1% SDS, 50 mM Tris (pH 7.4), 1 mM EDTA to equilibrate the gel bed. The amount of resin used was 1 μl of bed volume/2 μg of starting protein. 100 μl of resuspended protein from HeLa cells or MDCK cells stably expressing GYH generated in the previous step were added to an affinity column with 7 μl of 50% NH4OH (pH 7.4) (Sigma); the final NH4OH concentration was 1 M, and a separate 100-μl aliquot of resuspended protein sample was added to an affinity column without addition of NH4OH. The protein samples were incubated in the affinity columns by gentle inversion at room temperature overnight. Flow-through fractions from the affinity columns were collected. Unbound proteins were removed by successive washes of 20 bed volumes of 1% SDS, 50 mM Tris (pH 7.4), 1 mM EDTA, and corresponding fractions were collected for SDS-PAGE and or Western blot analyses. Specifically bound, formerly palmitoylated proteins were eluted with 100 mM β-mercaptoethanol (β-ME) in 1% SDS, 50 mM Tris (pH 7.4), 1 mM EDTA.

**Validation of PICA Using ICAT Reagents: Differential Labeling, Capture, and Identification of DHHC2 Substrates**—Proteins from untreated and siRNA-treated HeLa cells were extracted and treated with MMTS as above. Precipitated proteins from normal control cells and siRNA-treated cells were dissolved in 100 μl of fresh labeling buffer (0.05% SDS, 50 mM Tris (pH 7.4), 5 mM EDTA, 6 M urea), and the protein concentration was measured to ensure that they were equal and no more than 4 mg/ml. Control and siRNA-treated protein samples were allowed to react with L and H ICAT reagents, respectively, in 7 μl of 50% NH4OH (pH 7.4). After labeling, equal quantities of the control and siRNA-treated protein samples were combined, fractionated by SDS-PAGE, and digested in-gel with trypsin. ICAT-labeled peptides from different gel fractions (molecular weight regions) were enriched by avidin purification and cleaved to remove biotin groups.

**Mass Spectrometry Analysis of Captured Peptides**—Capillary reversed-phase HPLC separation of ICAT-labeled palmitoylated protein digests was performed on a 15-μm i.d. 50-μm inner diameter PepMap C18 column (LC Packings, San Francisco, CA) in conjunction with an Ultimate Capillary HPLC System (LC Packings). On-line tandem MS analysis was accomplished using a hybrid quadrupole time-of-flight instrument (QSTAR, Applied Biosystems) equipped with a nanoelectrospray source and operated with Analyst QS 1.1 data acquisition software (Applied Biosystems). Tandem mass spectrometric data generated via the QSTAR were extracted using Analyst QS 1.1 software and searched against the International Protein Index human protein sequence database (version 3.08 with 50,007 protein entries, available from the European Bioinformatics Institute as of July 2005) assuming trypsin (which cleaves carboxy-terminal to arginine or lysine residues) as the digestion enzyme (allowing for one missed cleavage) using the Mascot (version 1.19.05, Matrix Science, Boston, MA) database search algorithm. L and H ICAT modification of cysteine, deamidation of asparagine and glutamine, amino-terminal protein acetylation, oxidation of methionine, and pyroglutamylation formation from amino-terminal glutamine-containing peptides were included as variable modifications in the Mascot database search. A mass accuracy of ±0.3 Da for both precursor and fragment ions was used in the search as well. In general, probability-based MOWSE (molecular

**Protein Sample Preparation**—HeLa cells or MDCK cells (ATCC number CCL-34) stably expressing the fusion protein GAP43-YFP-His6 (GYH), a well characterized palmitoylated protein (7, 8), were washed and scraped into cold PBS (pH 7.4, 1 mM EDTA) containing protease inhibitors (Pierce). Cell suspensions were homogenized on ice in a glass tissue grinder with 40 strokes, and protein concentration was measured to ensure that they were equal and no more than 4 mg/ml. Control and siRNA-treated protein samples were allowed to react with L and H ICAT reagents, respectively, in 7 μl of 50% NH4OH (pH 7.4). After labeling, equal quantities of the control and siRNA-treated protein samples were combined, fractionated by SDS-PAGE, and digested in-gel with trypsin. ICAT-labeled peptides from different gel fractions (molecular weight regions) were enriched by avidin purification and cleaved to remove biotin groups.

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weight search) scores corresponding to a significance threshold of $p < 0.05$ were considered for peptide identification. Top ranked ICAT-labeled peptides that did not exceed this value were also considered; however, they were validated by manual interpretation of their corresponding MS/MS spectra. Peptides identified from the Mascot database search were then quantified via the LCMS reconstuct tool incorporated within the Analyst software. H/L ratios were calculated using areas (combined areas for mutually shared isotopic masses) derived from the quantitation algorithm. Mascot search results were imported into Scaffold version 1_06_05 (Proteome Software, Portland, OR) to facilitate evaluation of annotated MS/MS spectra.

Co-overexpression of DHHC2 and CKAP4/p63—To confirm that DHHC2 can palmitoylate CKAP4/p63, we used an established method (2, 11) in which we transfected COS cells with plasmids containing cDNAs encoding DHHC2 and/or CKAP4/p63 using FuGENE 6 (Roche Applied Science). Twenty-four hours after the transfection, the cells were serum-starved for 30 min and then metabolically labeled with 0.5 mCi [3H]palmitic acid for 6 h. Cells were washed in PBS and then harvested in SDS sample buffer containing 62.5 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 10 mM DTT, and bromphenol blue. Equal quantities of proteins from each condition were separated by SDS-PAGE. The gels were fixed for 30 min in isopropanol/water/acetic acid (25:65:10). Autofluorographic images were generated by treating the gels with Amplify (GE Healthcare) for 30 min. The gels were dried and exposed to film at $-80^\circ$ C for 24–48 h.

Immunoprecipitation of Metabolically Labeled CKAP4/p63—After 60 h of siRNA or mock transfection, HeLa cells (one 10-cm dish for each condition) were serum-starved for 30 min and then metabolically labeled with [3H]palmitic acid for 6 h (1 mCi/10-cm cell dish). Following labeling, the cells were washed twice in the dish with ice-cold PBS and scraped from the dish in 500 μl of ice-cold 1% Triton X-100, PBS (pH 7.4), 1 mM EDTA containing protease mixture (100×, Pierce). The scraped cells were lysed further, and proteins were extracted from the membrane fraction by gentle rocking in this buffer for an additional 30 min at 4°C. The lysate was then centrifuged at 14,000 × g in a precooled centrifuge for 30 min. The cleared lysate was transferred to a new tube, and the pellet was discarded. Protein G Dynabeads (100 μl) (Invitrogen) were washed twice with washing buffer (0.1 M sodium acetate (pH 5.0)) and incubated with 2 μg of CKAP4/p63 antibody in 200 μl of washing buffer for 60 min at room temperature. Following coupling of the CKAP4/p63 antibody (anti-CLIMP-63, clone G1/296; Alexis Biochemicals) the complex was washed four times with washing buffer. 100 μl of the bead-antibody complex were added to the cell lysate supernatant and incubated overnight at 4°C. Following binding of the bead-antibody complex to CKAP4/p63, the immunoprecipitate was washed four times with 800 μl of ice-cold 1% Triton X-100 in PBS, 1 mM EDTA, then resuspended in 60 μl of elution buffer (5% SDS, 8 M urea in PBS (pH 7.4)), and mixed gently for 1 h at room temperature. The eluted proteins were separated by SDS-PAGE and analyzed by fluorography for CKAP4/p63 labeled by [3H]palmitic acid and Western blot for CKAP4/p63 protein abundance.

Immunolocalization of CKAP4/p63 in HeLa Cells: Control Versus ZDHHC2 Knockdown—Double-stranded siRNA targeting ZDHHC2 was purchased from Dharmaco. HeLa cells were trypsinized for 10 min at room temperature and centrifuged in growth medium (minimum Eagle’s medium with 10% heat inactivated fetal bovine serum, 1% antibiotic/antimycotic solution, and 1% L-glutamine), and the cell pellet was resuspended in serum-free medium at a density of 1 × 10^5 cells/ml. 200 μl of the cell suspension was then transferred to a sterile 2-mm cuvette with 14 μg of siRNA and electroporated at 160 V/500-microfarad capacitance using a Bio-Rad Gene Pulser Xcell. HeLa cells were fixed using ethanol/acetonitrile (1:1) for 90 min at room temperature, washed three times with 1× PBS, and incubated with mouse monoclonal anti-CKAP4/p63 antibodies (anti-CLIMP-63, clone G1/296 diluted 1:100; Alexis Biochemicals) and anti-pann-cadherin rabbit polyclonal antibody (diluted 1:100; Abcam) in PBS, 5% normal goat serum for 2 h at room temperature. Cells were then washed three times with PBS and further incubated in PBS, 5% normal goat serum for 2 h at room temperature with secondary antibodies: FITC-labeled goat anti-rabbit (diluted 1:1000; Invitrogen) and TRITC-labeled goat anti-mouse (diluted 1:1000; Jackson ImmunoResearch Laboratories). Following five additional washes with PBS, cells were examined on a Nikon TE2000 epifluorescence microscope. Controls included cells processed without primary and/or secondary antibodies.

Western Blot Analysis of the Ability of PICA to Capture GYH—Specifically bound proteins from MDCK cells expressing GYH were eluted from the affinity column with β-ME as described above. Proteins were separated by SDS-PAGE, and the gels were either silver-stained or electropholeted to nitrocellulose membrane for Western blot analysis. The membrane was blocked in Tris-buffered saline with 0.1% Tween 20 (TBST) with 10% milk for 2 h at 4°C and then incubated with rabbit anti-GFP primary antibody (diluted 1:1000; Clontech) in the blocking buffer overnight at 4°C. Following incubation in primary antibody, the membrane was washed extensively in TBST and incubated for 2 h in horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (diluted 1:10,000; Pierce). Following incubation in secondary antibody the membrane was washed extensively in TBST. Detection of antibody-labeled GYH was by enhanced chemiluminescence (Pierce); the exposure time was 1 min.

RESULTS

Evaluating PICA Efficiency and Specificity—We initially determined the specificity and efficiency of PICA to capture palmitoylated and potentially palmitoylated proteins using heterobifunctional thiol-reactive probes that were analogous to the ICAT reagents. The results are shown in Fig. 2. Fig. 2A demonstrates the successful removal of nonspecifically bound proteins from the affinity matrix by a series of stringent washes. By the last wash of the last set in the series (Fig. 2, lane 7), we did not detect any more nonspecifically bound proteins being washed from the column. Elution of specifically bound, formerly palmitoylated proteins with β-ME (Fig. 2B) shows that many proteins were present in the eluate and that they spanned a broad range of molecular weights.

After the free, reactive thiols are protected by modification with MMTS, palmitoyl-cysteine thioester bonds were selectively cleaved by neutral hydroxylamine (NH₂OH (pH 7.4)), yielding a distinct set of free, reactive thiols comprised of formerly palmitoylated cysteines. When the step of selectively removing palmitates with NH₂OH was omitted, capture of proteins on the affinity matrix was eliminated (Fig. 2C). The inability to capture proteins under these conditions demonstrated the ability of MMTS to block all free, reactive thiols in the first step; furthermore it demonstrated that the thioester bond linking palmitate to cysteine was stable during protein harvesting from cells as well as during the protection step with MMTS. Under our experimental conditions, MMTS has been shown not to react with nitrosothiols or existing disulfides (10).

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Capture of proteins using PICA was effectively eliminated by a 2-h pretreatment of HeLa cells with 100 μM 2-bromo-palmitate (2BP), a nonspecific, general inhibitor of palmitoylation, as shown in Fig. 2D, providing additional evidence for the specificity of PICA chemistry for palmitoylation versus other types of post-translational cysteine modifications. 2BP is a halogenated analog of palmitate that is believed to block the transfer of palmitate to a substrate rather than inhibiting fatty acid synthesis as is believed to be the case with cerulenin (12–14). To our knowledge, there is no evidence to suggest that 2BP inhibits other post-translational modifications of cysteines such as nitrosylation directly or that it removes NO once it is bound. Moreover, endogenous nitrosylated proteins cannot be easily captured and identified unless large quantities of NO donors are added into an in vitro system to artificially increase the abundance (9). Taken together, these data suggest that proteins isolated by PICA are palmitoylated.

To further demonstrate the specificity and utility of PICA, we captured a palmitoylated fluorescent protein, GYH, that is commonly used as a fluorescent reporter of palmitoylation in living cells. This protein is a fusion of the amino-terminal 16 residues of GAP43 (a well-characterized palmitoylation substrate) to the amino terminus of mYFP, a monomeric, spectral mutant of Aequorea GFP (7), and a hexahistidine affinity tag on the carboxyl terminus. GYH is localized to the plasma membrane (PM) of living cells by virtue of its palmitoylation on two cysteine residues close to the amino terminus (7, 8, 15, 16). Detection of GYH in the eluate by Western blot (Fig. 2E, lanes 8 and 9) confirmed the ability of PICA to capture a positive control protein stably expressed in cells. We were able to verify visually in live MDCK cells stably expressing GYH, prior to processing the proteins for PICA, that GYH had the expected plasmamembrane localization, thereby confirming that it was palmitoylated.

Identification of DHHHC2 Substrates—To identify substrates of DHHHC2 in HeLa cells, we reduced the level of endogenous ZDHHC2 mRNA expression using siRNA (Dharmacon) and used mass spectrometry to measure the resulting changes in palmitoylation with PICA and ICAT reagents (17), the H tag on peptides from the ZDHHC2 knockdown and the L tag on those from the control. After 48–60 h of siRNA exposure, mRNA expression was reduced 93% as measured by quantitative real-time PCR. We identified 50 H and L ICAT-labeled peptides, including some from proteins already known to be palmitoylated (supplemental Table 1). For most of the peptides identified, the H/L ratio did not deviate significantly from parity, indicating that they were not substrates of DHHHC2. However, a single ICAT-labeled peptide from the majority of GYH is released during elution (lanes 8 and 9). The membrane was probed with anti-GFP primary antibody (Clontech) followed by horseradish peroxidase-conjugated secondary antibody (Pierce). Detection was by enhanced chemiluminescence (Pierce); the exposure time was 1 min.
CKAP4/p63, a type II transmembrane domain protein known to be palmitoylated (18) (Fig. 3), had an H/L ratio that was reduced consistently in four PICA runs by an average of 35% in the ZDHHC2 knockdown cells versus control (mean H/L ratio $= 0.65; p = 0.001; n = 4$ complete independent PICA runs from cell culture to mass spectrometry runs), indicating

**Fig. 3.** Mass spectrometry identification of CKAP4/p63 peptides labeled by H and L ICAT reagents. **A**, ICAT-labeled tryptic peptides ($m/z$ 753.9 and 756.9) derived from a putative palmitoylated protein and detected by LC-MS/MS in the retention time range 46–49 min. Following a Mascot protein database search of the corresponding MS/MS spectrum, the sequence was determined to be SSSSSSASAAAAAAASSSASC*SR, a tryptic peptide of CKAP/p63 modified at the cysteine residue with the ICAT reagent. The specificity of the labeling reaction allows for selective derivatization of palmitoyl-cysteines by the ICAT reagent. The 9-Da mass difference ($m/z$ 3 for a triply charged species) provided by the heavy ICAT reagent can be observed for this particular ICAT pair, and any variation in mass spectral intensity correlates to changes in protein palmitoylation induced from the ZDHHC2 knockdown treatment. **B**, reconstructed ion chromatograms derived from the monoisotopic masses corresponding to the L and H ICAT-labeled peptide SSSSSSASAAAAAAASSSASC*SR from a separate experiment. Upon calculation of the peak area ratios (H/L) obtained from four separate experiments using a quantitation algorithm within the data acquisition software, this particular peptide showed a 35% decrease in palmitoylation ($p < 0.001, n = 4$ complete independent PICA runs from cell culture to mass spectrometry runs) after ZDHHC2 knockdown. **C**, MS/MS spectrum of a triply charged ion at $m/z$ 757.06. After a protein database search using Mascot, the peptide sequence was determined to be SSSSSSASAAAAAAASSSASC*SR (Mascot ion score of 64.6), a heavy ICAT-labeled tryptic peptide of CKAP/p63. The presence of the isobaric fragment ions $y_1/b_2$ and $y_2/b_3$ ($m/z$ 0.05) made unambiguous assignment difficult for these particular ions given the mass measurement accuracy specified for data processing and subsequent database searching. However, after inspection of the MS/MS raw data, adequate mass resolving power and peak shape were obtained to identify both $y_1$ and $b_2$ ions. The mass accuracy of the MS/MS spectrum allowed for putative assignment of the peak at $m/z$ 262 as the $y_2$ fragment ion, although the $b_3$ fragment ion should not be precluded because of limited resolving power and peak shape obtained at this $m/z$ value. Only one of the four data sets for the identification of CKAP4/p63 is shown here. It is representative of the other three. cps, counts/s.
that it was a substrate of DHHC2, CKAP4/p63 was palmitoylated on Cys100 (indicated by the asterisk) in the tryptic peptide 78SSSSSSASAAAAAAAASSSASC*SR102, corresponding to the cysteine previously reported to be palmitoylated (18). siRNA-mediated ZDHHC2 knockdown did not change the abundance of CKAP4/p63 protein versus control ( nonsense siRNA, Oligofectamine-treated) as determined by Western blot analyses of CKPA4/p63 (supplemental Fig. 2). Triply charged ions corresponding to H and L ICAT-labeled tryptic peptides of CKAP4/p63 were observed at m/z 754.05 and 757.06, respectively (Fig. 3A). The Mascot database search of MS/MS spectra obtained for these peptides confirmed the identity of CKAP/p63 with high confidence. Mascot ion scores resulting from multiple MS/MS spectra acquired for both the L and H ICAT-labeled CKAP/p63 tryptic peptide consistently exceeded the identity score defined at p < 0.05. Fig. 3C displays a representative MS/MS spectrum of an ICAT-derivatized CKAP/p63 peptide derived from the ZDHHC2 knockdown sample (H labeled). The Mascot ion score of 64.6 greatly surpassed the identity score of 34.2 in this particular example. The total protein coverage obtained from this analysis was 4% based on one peptide identification. PICA selectively isolates and captures palmitoyl cysteine-containing tryptic peptides and excludes all others (because they are not modified by ICATs), and thus, the total number of peptides that can be used for identification is limited only to putative, palmitoyl-cysteine-containing peptides.

Confirmation That CKAP4/p63 Is a Substrate of DHHC2—CKAP4/p63 was already known to be palmitoylated on the cysteine that we identified as having reduced palmitoylation in response to ZDHHC2 knockdown (19). The consistently reduced H/L ratio in response to ZDHHC2 knockdown for this palmitoyl peptide in four consecutive PICA runs indicated that it was a substrate for DHHC2. We confirmed this finding using two independent methods. First we overexpressed ZDHHC2 and/or CKAP4/p63 in COS cells and metabolically labeled proteins with [3H]palmitate (2, 20). When both ZDHHC2 and CKAP4/P63 were overexpressed together, we measured a 5.7-fold increase in the incorporation of [3H]palmitate into CKAP4/p63 (Fig. 4 lane 4) versus labeling of endogenous CKAP4/p63 by endogenous DHHC2 (Fig. 4, lane 1). Overexpression of CKAP4/p63 alone or ZDHHC2 alone resulted in slightly elevated levels of [3H]palmitate incorporation (Fig. 4, lanes 2 and 3). Treatment of the cellular protein extracts with hydroxylamine after metabolic labeling immediately prior to separation by SDS-PAGE hydrolyzed the thioester bond and released [3H]palmitate from CKAP4/p63 confirming that [3H]palmitate is attached by a thioester bond (lane 5). Equal amounts of total protein lysate were added to each lane. The fluorographic signal was the result of a 24-h exposure. The integrated signal density of each band was measured using ImageJ (42) and normalized to the mock-transfected control, lane 1.

FIG. 4. Confirmation that CKAP4/p63 is a substrate of DHHC2. Co-overexpression of CKAP4/p63 and ZDHHC2 in COS cells resulted in a 5.7-fold increase in [3H]palmitate labeling of CKAP4/p63 (lane 4) versus control (lane 1) confirming that CKAP4/p63 is a substrate of DHHC2. Overexpression of ZDHHC2 alone (lane 2) or CKAP4/p63 alone (lane 3) resulted in much smaller signal increases versus control. Treatment of the protein sample run in lane 4 with hydroxylamine removes [3H]palmitate from CKAP4/p63 confirming that [3H]palmitate is attached by a thioester bond (lane 5). Equal amounts of total protein lysate were added to each lane. The fluorographic signal was the result of a 24-h exposure. The integrated signal density of each band was measured using ImageJ (42) and normalized to the mock-transfected control, lane 1.
in which ZDHHC2 expression was silenced, the distribution of CKAP4/p63 was restricted to ER that had moved close to the nucleus (Fig. 5). This finding indicates that palmitoylation is required for trafficking of CKAP4/p63 from the ER, its most highly characterized subcellular residence (19, 24, 25), to the PM and that blocking palmitoylation of CKAP4/p63 may result in a restructuring of the ER.

**DISCUSSION**

Here we describe the development and validation of PICA, a novel method that identifies substrates of individual PATs without prior knowledge of their relationship. Using PICA we identified CKAP4/p63 as a physiologically important substrate of DHHC2, the PAT encoded by ZDHHC2. At least five genes encoding PATs have been implicated in human disorders, including schizophrenia (ZDHHC8) (26), Huntington disease (ZDHHC17 (HIP14)) (27, 28), X-linked mental retardation (ZDHHC15 and ZDHHC9) (29, 30), and cancer (ZDHHC2 and ZDHHC17) (4, 28). ZDHHC2 was originally named REAM (reduced expression in metastasis) (4) and maps to chromosome 8 (p21.3-22), a region that is frequently deleted in many types of metastatic cancer, including colorectal cancer (31–33), hepatocellular carcinoma (32, 34), nonsmall cell lung cancers (32, 35), and cancers of the breast (36, 37), urinary bladder (38), and prostate (39). The absence of ZDHHC2 expression in many highly metastatic cancers suggested to us that identification of a hypo/depalmitoylated substrate of DHHC2 would provide important insight into the molecular mechanisms underlying metastasis.

CKAP4/p63 was identified previously as a palmitoylated protein that physically links the ER to microtubules (25, 40, 41). More recently it was identified as a cell surface receptor for tissue plasminogen activator (23), surfactant protein A (22), and most recently, APF (21). APF is a sialoglycopeptide inhibitor of bladder epithelial cell proliferation that is secreted by bladder epithelial cells from patients with interstitial cystitis (43, 44). It was found to reversibly inhibit the growth of bladder epithelial cells in vitro (44, 45), to profoundly inhibit the proliferation of both normal bladder epithelial cells and bladder carcinoma cells in vitro (43–45), and to inhibit proliferation of HeLa cells, a cervical carcinoma cell line (21), with an IC50 (1 nM) approximately equal to its IC50 for primary bladder cells (21). The discovery that CKAP4/p63, the receptor for APF, is a substrate of DHHC2, a putative tumor/metastasis suppressor, provides an important link between the proliferative properties of many types of cancer cells and the reduced expression or mutation of ZDHHC2.

The loss of CKAP4/p63 localization on the PM in response to siRNA-mediated silencing of ZDHHC2 expression (Fig. 5) provided further evidence that CKAP4/p63 is a substrate of ZDHHC2 as well as elucidating the role of palmitoylation in regulating the ability of CKAP4/p63 to function as a cell surface receptor. Although immunolocalization did not provide a
quantitative measure of the decrease in CKAP4/p63 palmitoylation, it did provide a qualitative, observable link between the activity of DHHC2 and its substrate CKAP4/p63. CKAP4/p63 was first described as a palmitoylated transmembrane protein localized predominantly to the ER that physically links the ER to the microtubular network in a phosphorylation-dependent manner. In our control experiments (mock or non-sense siRNA-transfected HeLa cells) normally palmitoylated CKAP4/p63 was expressed on endomembranes that extended throughout the cytoplasm as well as on the PM (Fig. 5). Palmitoylation-dependent trafficking of other, PM-localized, transmembrane receptors (46, 47) is also known to be required for export or trafficking from the ER to the PM. Immunolabeling of CKAP4/p63 HeLa cells following ZDHHC2 knockdown also revealed what appeared to be a remodeling of the ER from an extensive membrane system occupying much of the interior of the cell into a tight ring around the nucleus. The staining pattern for CKAP4/p63 that we observed in response to ZDHHC2 knockdown was remarkably similar to the pattern observed by Vedrenne et al. (25) in cells expressing either deletion or phosphomimicking mutants of CKAP4/p63 that could not bind microtubules. For cells expressing these mutants, they reported that the ER collapsed around the nucleus while leaving the microtubular network intact and suggested that CKAP4/p63-mediated stable anchoring of the ER to microtubules is required to maintain the spatial distribution of the ER. Our data suggest that blocking palmitoylation also reduces the interaction of CKAP4/p63 with microtubules similarly to (and potentially via) phosphorylation. This observation is also supported by our finding that palmitoylated CKAP4/p63 binds significantly more strongly to microtubules than does unpalmitoylated CKAP4/p63 in in vitro microtubule binding assays.

Following transfection of cells with siRNA, ZDHHC2 mRNA was reduced by 93% relative to control indicating high transfection and knockdown efficiency, whereas palmitoylation of CKAP4/p63 was reduced by an average of 35% as measured by PICA. This disparity may suggest that DHHC2 has a long half-life relative to the mRNA that encodes it. Alternatively CKAP4/p63 may be the substrate of more than one PAT as is the case for other palmitoyl substrates (2, 20). If so, whether this represents a naturally occurring redundancy in PAT-substrate specificity or a compensatory adaptation resulting from the reduction in DHHC2 abundance is not clear. Although the residence half-life of palmitate on several known palmitoyl proteins is ~3 h (8, 48, 49), much shorter than the time course of these experiments, it is also possible that CKAP4/p63 palmitoylation has a much longer half-life (50).

De novo identification of the substrates of individual PATs in live mammalian cells is the principle, novel strength of PICA and an important addition to the current set of methods used to study palmitoylation. In addition to identifying substrates of individual PATs, PICA identifies other palmitoylated and potentially palmitoylated proteins. With PICA, protein samples from control cells and from cells in which ZDHHC2 expression has been knocked down are differentially labeled by H and L ICATs. In these samples, potentially all formerly palmitoylated cysteines in all peptides are labeled whether or not the cysteine was a substrate for DHHC2-mediated palmitoylation. In our experiments, the average H/L ratio of all peptides was 1.03 (S.D. = 0.09) indicating that most peptides were not substrates of DHHC2 (supplemental Table 1); those ≥1 were not considered to be substrates of DHHC2, whereas CKAP4/p63 had an average H/L ratio of 0.65 in four complete, independent PICA runs indicating that it was in fact a substrate of DHHC2. The H/L ratios of other proteins such as glycerolaldehyde-3-phosphate dehydrogenase (supplemental Table 1) were reduced but did not reach the statistical threshold to qualify as a substrate that we established when the study began. Such potential substrates are currently being evaluated further to establish their relationship with DHHC2. It is possible that PICA may return false-positive hits in terms of whether or not a protein is simply palmitoylated, but even if PICA falsely identified a non-palmitoylated protein as being one that is palmitoylated, the H/L ratio would be very close to 1 because it is not a substrate of DHHC2 and as such would have an equal chance of being captured in both control and ZDHHC2 knockdown cells. So for the identification of substrates of a single PAT using PICA, the possibility of a false-positive result is low. Confirmation of the PAT-substrate relationship by co-overexpression of ZDHHC2 and CKAP4/p63 combined with metabolic labeling, the immunoprecipitation of metabolically labeled CKAP4/p63 from knockdown and control cells, and the physical relocation of CKAP4/p63 in response to ZDHHC2 knockdown that we determined by immunocytochemistry provided additional support for the utility and accuracy of the PICA in the identification of PAT substrates. Western blot confirmed that ZDHHC2 knockdown did not alter the abundance of CKAP4/p63 relative to actin in cells (supplemental Fig. 2). Likewise the mean H/L ratio of ~1 for all palmitoylated and potentially palmitoylated proteins indicated that there was no change in overall protein expression level as a result of ZDHHC2 knockdown. Mass spectrometric data from two peptides with essentially unchanged H/L ratios following ZDHHC2 knockdown (supplemental Fig. 1) illustrate the contrast in results between peptides that are substrates of DHHC2 (Fig. 3) and proteins that are possibly palmitoylated but not substrates of DHHC2.

Selective capture of ICAT-labeled palmitoyl peptides, rather than capturing, trypsinizing, and analyzing whole proteins, significantly reduced the complexity of the sample thereby simplifying subsequent analysis by mass spectrometry. This selective isolation of ICAT-labeled (potentially formerly palmitoylated) tryptic peptides with PICA allowed us to imme-

3 S. L. Planey and D. A. Zacharias, unpublished data.
diately identify the palmitoyl-cysteine(s) in proteins that often contain many cysteines.

An analogous, complementary proteomics method that exemplifies the strategic advantages of using a yeast model published by Roth et al. (1) was used to link specific palmito-
ylated proteins to the activity of several of the seven yeast PATs. Several differences exist between PICA and the study by Roth et al. (1). PICA identifies PAT-substrate partners in mammalian cells using siRNA-mediated gene knockdown, a method that is typically not as robust as completely eliminating the expression of a single PAT over many cell generations as was done in yeast (1). Therefore, the magnitude of the resulting reduction in substrate palmitoylation is not expected to be as great using siRNA in mammalian cells. The work by Roth et al. (1) used spectral counting or multidimensional protein identification technology (51) to identify palmitoylated proteins. This method may identify less abundant proteins than PICA because more than a single peptide is used to identify a palmitoyl protein. By contrast in this respect, PICA provides the advantage of specifically identifying the palmit-
yloyl-cysteine(s) within a palmitoyl protein allowing a rapid transition to follow-up experiments to evaluate the specific role of the palmitoyl-cysteine(s) (versus the many non-palmi-
tyol-cysteines existing in the protein) to the function of the protein and downstream signaling pathways.

Our analyses suggest that PICA detects what are likely to be the more abundantly expressed palmitoyl (and putative palmitoyl) proteins in HeLa cells. We are currently evaluating the relative sensitivity of PICA as described here versus the identification of palmitoyl proteins using multidimensional protein identification technology analysis of proteins captured by PICA. Finally PICA also provides a mechanism for the quantitative analysis of the effects of pharmacological mod-
ulation of PATs and for the consequences of their altered function due to mutation and altered expression of the genes that encode them.

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[6] A Partner in Research with Nikon. To whom correspondence
should be addressed: The Whitney Laboratory for Marine Bioscience,
University of Florida, St. Augustine, Florida 32080. Tel.: 904-461-
4021; Fax: 904-461-4052; E-mail: daz@whitney.ufl.edu.
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