DHHC Protein S-Acyltransferases Use Similar Ping-Pong Kinetic Mechanisms but Display Different Acyl-CoA Specificities

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Background: DHHC protein acyltransferases catalyze palmitoylation by a poorly characterized mechanism.

Results: The acyl group covalently attached to the enzyme is transferred to substrate. Long chain acyl-CoAs are better substrates for DHHC2 than DHHC3.

Conclusion: DHHC proteins share a two-step ping-pong mechanism but display different acyl-CoA substrate specificity.

Significance: Acyl-CoA analogs and mechanism-based inhibitors represent possible strategies for DHHC inhibition.

DHHC proteins catalyze the reversible S-acylation of proteins at cysteine residues, a modification important for regulating protein localization, stability, and activity. However, little is known about the kinetic mechanism of DHHC proteins. A high-performance liquid chromatography (HPLC), fluorescent peptide-based assay for protein S-acylation activity was developed to characterize mammalian DHHC2 and DHHC3. Time courses and substrate saturation curves allowed the determination of $V_{\text{max}}$ and $K_m$ values for both the peptide N-myristoylated-GCG and palmitoyl-coenzyme A. DHHC proteins acylate themselves upon incubation with palmitoyl-CoA, which is hypothesized to reflect a transient acyl enzyme transfer intermediate. Single turnover assays with DHHC2 and DHHC3 demonstrated that a radiolabeled acyl group on the enzyme transferred to the protein substrate, consistent with a two-step ping-pong mechanism. Enzyme acylation and acyltransfer to substrate displayed the same acyl-CoA specificities, further supporting a two-step mechanism. Interestingly, DHHC2 efficiently transferred acyl chains 14 carbons and longer, whereas DHHC3 activity was greatly reduced by acyl-CoAs with chain lengths longer than 16 carbons. The rate and extent of autoacylation of DHHC3, as well as the rate of acyl chain transfer to protein substrate, were reduced with stearyl-CoA when compared with palmitoyl-CoA. This is the first observation of lipid substrate specificity among DHHC proteins and may account for the differential S-acylation of proteins observed in cells.

Protein S-acylation is the post-translational addition of long chain fatty acids to cysteine residues via a thioester linkage. Unlike other lipid modifications, S-acylation is reversible and thus regulated via acylation/deacylation cycles in cells. This regulation is important for the activity and localization of key signaling proteins including Ras isoforms (1–3), G-protein α-subunits (4), huntingtin (5, 6), endothelial nitric oxide synthase (7), and ion channels (8). Protein acyltransferases (PATs)3 catalyze the addition of fatty acids to proteins, whereas acyl-protein thioesterases remove them. Despite the importance of protein S-acylation in these signaling pathways and in human diseases (9), little is known about the kinetic mechanism, regulation, and substrate specificities of PATs and acyl-protein thioesterases.

Genetic and biochemical studies in yeast have established that a family of integral membrane enzymes known as DHHC proteins catalyzes protein S-acylation. Although Saccharomyces cerevisiae have seven DHHC PATs, mammalian genomes encode at least 23. DHHC proteins are named for a highly conserved Asp-His-His-Cys sequence within a larger cysteine-rich domain (DHHC-CRD) that is situated on the cytoplasmic face of the membrane between four transmembrane domains (10).

In vitro analyses with radiolabeled palmitoyl-CoA (palmCoA) have demonstrated that DHHC proteins are sufficient to catalyze the transfer of fatty acids from CoA to cysteine residues within target protein substrates. Additionally, DHHC proteins themselves become acylated upon incubation with palmCoA, a process called enzyme acylation. Mutational analysis has revealed that the cysteine residue within the DHHC motif is indispensable for both palmitoyl transfer and autoacylation activities (11, 12); however, the site of autoacylation remains unknown as well as whether autoacylation occurs in cis or in trans. It has been hypothesized that DHHC autoacylation reflects a transient acyl enzyme intermediate with DHHC proteins using a two-step ping-pong mechanism to catalyze transfer (11, 12).

Alternatively, DHHC autoacylation may reflect a modification of the enzyme that is not transferred to substrate but serves another function.

3 The abbreviations used are: PAT, protein acyltransferase; DHHC, Asp-His-His-Cys; DDM, n-dodecyl-b-D-maltoside detergent; palmCoA, palmitoyl-CoA; [3H]palmCoA, [3H]-palmitoyl-CoA; TCEP, tris(2-carboxyethyl)phosphine; 16-12-NBD-PC, 1-palmitoyl-2-{12-[((7-nitro-2-1,3-benzo-xadiazol-4-yl)amino)dodecanoyl]-sn-glycero-3-phosphocholine; NBD, nitrobenzoxadiazole; DMSO, dimethyl sulfoxide.
The fatty acid attached during S-acylation is most often the saturated 16-carbon fatty acid palmitate (C16:0); thus, the process is frequently called S-palmitoylation, or simply, palmitoylation. However S-acylation of other chain lengths has been reported. Incubation of platelets with [3H]arachidonate (C20:4) resulted in the labeling of endogenous G-protein subunits αs, αt, αt2, and αi, via a thiorester linkage (13). Mass spectrometry of fatty acids attached to native rhodopsin revealed that ~83% are C16:0, whereas the remaining are a mixture of 14:0, 15:0, 16:1, 18:0, 18:1, 18:2, 20:4, and 22:6 (14). Metabolic radiolabeling with C16:0 versus either C20:4 arachidonate (15) or C18:0 sterate (16) demonstrated that some proteins are preferentially modified with chain lengths other than C16:0. More recently, using click chemistry techniques and alkyl fatty acids that mimic myristate, palmitate, or stearate to enrich for acylated proteins, Hang and co-workers (17) identified proteins from Jurkat T cells that were selectively labeled with different chain lengths. The mechanism responsible for these differences in acyl chain length attachment remains unclear.

In the present study, we characterize the kinetic mechanism and lipid substrate specificity of DHHC proteins. A high-performance liquid chromatography (HPLC) fluorescent peptide-based PAT assay was developed to measure rate constants for two representative DHHC proteins. Single turnover experiments directly addressed the question of whether DHHC auto-acylation is a transient acyl enzyme intermediate in a two-step ping-pong mechanism. One corollary of this predicted reaction scheme is that acyl chain lengths capable of being transferred to protein substrates should also be capable of autoacylating the DHHC protein. This prediction was tested as well as the mechanism of DHHC lipid substrate specificity.

**EXPERIMENTAL PROCEDURES**

Reagents—The fluorescent peptide myrGCG4 was synthesized by AnaSpec, Inc. (San Jose, CA) and consists of N-myristoylated glycine, cysteine-protected by a disulfide link tert-butyl group, and glycine-linked via ethylenediamine to toyl group, and glycine-linked via ethylenediamine to {12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl}-CoA was separated from free fatty acid by chloroform/methanol extraction (19). Acetonitrile was purchased from Honeywell. Internal standard 16-12-NBD-PC (1-palmitoyl-2-[12-17-nitro-2-1,3-benzoxadiazol-4-yl]amino) dodecanoyl)-sn-glycero-3-phosphocholine) and nonradiolabeled C6- and C10-CoAs were from Avanti Polar Lipids. CoA and the other unlabeled acyl-CoAs were from Sigma. Immunoblots were performed with mouse anti-FLAG M2 (Sigma) (1:2000) and goat anti-mouse IgG conjugated to HRP (MP Biomedicals) (1:1000). Proteins were detected using enhanced chemiluminescence and imaged with a VersaDoc™ 5000 MP (Bio-Rad) for 5 min. Images were generated by fixing the γ setting at one and adjusting the high-low settings uniformly for the entire image.

**HPLC-based Fluorescent Peptide PAT Assay**—The peptide deprotection, integrity, and PAT assays were analyzed on a reversed phase Vydac C4 (5-μm, 300 Å, 4.6 x 250-mm) column using a Beckman Coulter Gold HPLC system (508 autosampler, 126NM solvent module, 166N detector) inline with a JASCO FP2020 fluorescence detector. Separation was achieved using acetonitrile gradients generated from buffer A (20% acetonitrile, 80% water, 0.1% trifluoroacetic acid) and buffer B (100% acetonitrile).

Peptide (0.4 mm, 156 μl) was deprotected by overnight incubation in 75% N,N-dimethylformamide, 160 mM β-mercaptoethanol, 80 mM DTT, and 100 mM HEPES, pH 8.0, at 50 °C in the dark under argon. Deprotected peptide was collected by extraction with the sequential addition of 100 μl of methanol, 800 μl of dichloromethane, and 800 μl of water. Three 800-μl extractions of dichloromethane were pooled and dried under N2. Extracts were dissolved in methanol and transferred to an HPLC vial. Aqueous DTT was added to give a final concentration of 70% methanol, 10 mM DTT. The peptide solution was incubated at room temperature for 1 h to reduce disulfide-linked peptide dimers. The reduced peptide was separated from DTT and other contaminants by HPLC. Fractions containing fluorescent peptide were manually collected, dried under N2, dissolved in methanol, aliquoted, redried, and stored at ~70 °C under argon. One aliquot was dissolved in 2-propanol, and A461.5 was measured to quantify peptide concentration using a molar absorptivity of 29,056 M⁻¹ cm⁻¹.

Prior to a peptide PAT assay, peptide dimers were reduced by incubation in 69% DMSO, 0.14% DDM, and 1.7 mM tris(2-carboxyethyl)phosphine (TCEP) for 3 h under argon, protected from light at 25 °C. Greater than 90% of the peptide was the deprotected and monomeric species as assessed by HPLC before each experiment. The reduced peptide was diluted with aqueous buffer to 25% DMSO, 0.05% DDM, 0.75 mM TCEP, and 50 mM MES, pH 6.4. PalmCoA (and later, other acyl-CoAs) dissolved in 20 mM MES 6.4 and 0.01% DDM and peptide were mixed in glass test tubes and warmed to 25 °C. Purified DHHC protein warmed to 25 °C was added to start the reaction. The final reaction was 50 μl at 50 mM MES, pH 6.4, 10% DMSO, 0.3 mM TCEP, and 0.028% DDM. Final concentrations of DHHC, acyl-CoA, and myrGCG and reaction times are noted in the figure legends. Reactions were stopped with 500 μl of dichloromethane and held on ice until all reactions were complete. The reactions were spiked with 250 μl of 0.02 μM 16-12-NBD-PC dissolved in methanol as an internal standard and 250 μl of aqueous buffer (50 mM MES, pH 6.4, 250 mM NaCl) to cause phase separation. This internal standard was chosen because it was tagged with NBD similar to the peptide, does not overlap with other peaks, and elutes in the same solvent as the pali-

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4 Throughout this study, myrGCG refers to N-myristoylated tripeptide Gly-Cys-Gly ethylenediamine linked to fluorescent NBD; myrLCαt refers to N-myristoylated lymphocyte specific kinase, N-terminal residues 1–226; and myrGαs1 refers to N-myristoylated G-protein α-subunit 1.
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toylated peptide, minimizing the high solvatochromic shift of NBD (20). The lower organic phase was collected, and the aqueous phase was extracted twice more with 500 µl of dichloromethane. Pooled extracts were clarified with 300 µl of methanol, dried under N₂, and stored at −20 °C. For HPLC analysis, the samples were dissolved in 100 µl of isopropyl alcohol and 100 µl of 0.5 mM TCEP added to reduce disulfide-linked peptides. For each reaction, an aliquot of 50 µl was injected onto a reversed phase C4 column equilibrated in 35% buffer B at 1 ml/min. After 1 min, a linear gradient over 5 min increased the mobile phase to 82.5% B, and it was held there for 10 min. The mobile phase was then returned to 35% B over 1.5 min and allowed to equilibrate for 3.5 min (see Fig. 1A, dashed line). UV absorbance was recorded at 260 nm, fluorescence was excited at 465 nm, and emission was recorded at 531 nm with the gain set at 10–100. This HPLC method was adapted from work by others (21). The 32 Karat software, Version 8, was used to record data and determine the area under the curve for peptide and internal standard peaks. Areas were converted to pmol of acylated myrGCG, fit to the Michaelis-Menten equation using nonlinear regression, and plotted with Prism 5 (GraphPad Software, Inc.).

For directly monitoring transfer of various acyl-CoA chain lengths, a similar assay was used with 10 µM acyl-CoAs and 5 µM protected myrGCCG in a final 50-µl reaction. The addition of DHHC (10 nM) or buffer was used to start the reaction, which was incubated at 25 °C for 10 min. Reactions were stopped with 500 µl of dichloromethane and processed as described above.

 Constructs, Expression, and Protein Purification—Plasmids for murine myrLckNT and human DHHC2 were described previously (18). Mouse DHHC3 was amplified from the cDNA (Image Clone 3669723, NM_026917.4) and subcloned into pBlueBac4.5 (Invitrogen) to encode a protein with a FLAG-His₆ sequence (GSELRQAYVVDYKDIDDDKNSAEFHHHH-HH(stop)) appended to the C terminus of DHHC3. Called pML1117, this plasmid was used to generate catalytically inactive DHH53-FLAG-His₆ (pML1354) by site-directed mutagenesis (Stratagene) of Cys-157. Recombinant baculoviruses were generated as described previously (18). DHHC2 and DHHC3 WT and DHH53 mutants were expressed in Sf9 cells and purified by nickel-nitritolriacetic acid metal chelate and FLAG affinity chromatography as described previously except that TriEx™ S9 cells and medium (EMD Chemicals) were used for cell culture. The concentration of enzyme was determined by extrapolation from a linear curve with known concentrations of bovine serum albumin using SYPRO® Ruby protein gel stain (Lonza, Rockland, ME) and quantitation using a VersaDoc™ 5000 imaging system. Nickel-nitritolriacetic acid elutions were used without a second step of purification for acyl-CoA competitions (see Fig. 3). N-Myristoylated G-protein α1 (myrGα11) and myrLckNT were co-expressed in Escherichia coli with N-myristoyltransferase and purified as C-terminal His₆-tagged proteins using established protocols (18, 22).

Direct Transfer from DHHC to Protein Substrate—For single turnover experiments (see Fig. 2), 350 pmol of DHHC protein was incubated with 40 µl of FLAG affinity resin equilibrated in buffer C (50 mM Tris, pH 7.4, 100 mM NaCl, 0.06% DDM, 5% glycerol, and 1 mM EDTA) at 4 °C with end-over-end rotation for 60 min. Bound DHHC was washed three times with 500 µl of buffer C and twice with 650 µl of buffer D (50 mM MES, pH 6.4, 20 mM NaCl, 0.06% DDM, 5% glycerol, and 1 mM EDTA). Bound DHHC was autoacylated by incubation with buffer D containing [³H]palmCoA (1400 pmol) for 7 min on ice. Free [³H]palmCoA was removed by washing with 850 µl of buffer D 11 times until the radioactive acyl-CoA content of the washes was <5 nM. Radiolabeled DHHC was eluted eight times with 50 µl of buffer D containing 0.3 µg/µl 3×FLAG peptide (Sigma). Elutions 2–8 were pooled, and single turnover time courses were initiated within 1 h of elution by the addition of 1 µM unlabeled palmCoA and either protein substrate or buffer at 25 °C. Aliquots were removed at the indicated times and stopped with 5× sample buffer with 2 mM TCEP final. Reactions were divided between two SDS-PAGE gels with one set of gels being processed for fluorography and the other for scintillation counting using established methods (18). For single turnover experiments with stearyl (see Fig. 5, C and D), an identical procedure was followed except that [³H]stearyl-CoA was used.

Acyl-CoA Specificity of Autoacylation and Transfer—For competition PAT assays, [³H]palmCoA and unlabeled acyl-CoA were premixed on ice. Likewise, DHHC and protein substrate were premixed before adding to the lipid substrate mixture and incubating 6 min at 25 °C. Final assay concentrations were 20 nM DHHC, 1 µM [³H]palmCoA, 10 µM competing acyl-CoA, and either 1 µM myrGα11 or 0.5 µM myrLckNT in a total of 25 µl. Assays were stopped with 5× sample buffer with 2 mM TCEP final and processed for scintillation counting.

Hydrolysis of acyl-CoAs (see Table 2) was determined by incubating DHHC2 or DHHC3 (45 nm) or buffer with either [³H]palmCoA or [³H]stearyl-CoA at 25 °C and stopping with SDS. An aliquot of the reaction, equivalent to 5 pmol of the initial acyl-CoA in the reaction, was spotted on thin layer chromatography plates (LK6DF silica gel, Whatman) and resolved with 50% n-butanol, 20% acetic acid, 30% water for 5 h. The plate was dried, sprayed twice with EN3HANCE™ spray surface autoradiography enhancer (PerkinElmer Life Sciences), and exposed to film at −70 °C for 42 h. The regions of the TLC plate containing free fatty acids were located by comparison with the film, scraped, and quantitated with scintillation counting. Background free fatty acid was determined from a control reaction lacking DHHC and subtracted before fitting the data by linear regression. To the remaining reaction, 5× sample buffer was added, and an aliquot representing 1 pmol of DHHC protein was resolved on SDS-PAGE gels and processed for scintillation counting to monitor enzyme autoacylation. Analyzing a reaction without DHHC was set as the zero time point allowing the data to be fit to a single phase exponential by nonlinear regression.

RESULTS

Determination of DHHC3 and DHHC2 Kinetic Constants with Fluorescent Peptide, HPLC-based PAT Assay—To characterize DHHC proteins mechanistically, we determined steady state kinetic parameters with a modified HPLC method initially described for the characterization of PAT activity in cell lysates (21). The advantages of the HPLC-based peptide assay were
Ease of achieving saturating substrate concentrations and elimination of the need for radioactive acyl-CoA compounds. For a palmitoylation site mimic, the three-residue peptide glycine-cysteine-glycine was synthesized with the N terminus myristoylated and the C terminus linked to the fluorescent group NBD (myrGCG; Fig. 1A, inset). A previous study showed that myrGCG is taken up by cells and efficiently palmitoylated in a manner that is time-dependent and saturable (23), suggesting that it is a good substrate for in vitro palmitoylation assays.

DHHC2, DHHC3, and DHHC9/GCP16 were individually tested for their ability to palmitoylate myrGCG. DHHC2 and DHHC3 showed similar levels of palmitate incorporation, 2.5 times that of DHHC9/GCP16. In vivo evidence suggests that DHHC2 and DHHC3 have broad protein substrate specificity (9). Both expressed well in insect cells infected with recombinant baculovirus and were purified to near homogeneity using nickel chelate and FLAG affinity chromatography (supplemental Fig. S1). Therefore, they were chosen for further kinetic analysis. To determine the linear range of the reaction, a time course was performed. DHHC3, palmCoA, and myrGCG were incubated for various times, spiked with an internal standard, and separated on a C4 reversed phase HPLC column monitored with a fluorescence detector. A representative chromatogram is shown in Fig. 1A. In addition to peaks of unreacted and palmitoylated peptide, a peak corresponding to a disulfide-linked myrGCG dimer appears, which results from the drying of unprotected myrGCG in the absence of reducing agent. The area of the palmitoylated peptide peak was compared with that of the internal standard and converted to pmol of palmitoylated peptide. At low concentrations of both substrates, the reaction was linear for at least the first 6 min (Fig. 1B); however, the standard assay was limited to 4 min before depletion of either substrate occurred. To determine $K_m$ and $V_{max}$ values, one substrate concentration was held at a constant saturating concentration, whereas the other varied. Saturation with palmCoA and analysis with nonlinear regression revealed a $V_{max}$ of 9.9 pmol/min/pmol of DHHC3 and a $K_m$ of 1.3 $\mu$M myrGCG (Fig. 1C, Table 1). At saturating myrGCG, a $V_{max}$ of 11.9 pmol/min/pmol and a $K_m$ of 5.9 $\mu$M palmCoA were observed (Fig. 1D). For both peptide and palmCoA titration curves, $V_{max}$ values were

* B. C. Jennings and M. E. Linder, unpublished results.

**FIGURE 1.** Fluorescent peptide, HPLC-based kinetic characterization of DHHC3 and DHHC2. A, structure of the tripeptide substrate N-myristoylated Gly-Cys-Gly tagged via ethylenediamine with fluorescent NBD (myrGCG, inset). The elution profile of myrGCG following reversed phase chromatography is shown. myrGCG was incubated with palmCoA and DHHC3 in vitro. The reaction was spiked with internal standard (16-12-NBD-PC), extracted, dried, and chromatographed using the gradients (dashed line) shown with buffer A (20% acetonitrile, 80% water, 0.1% TFA) and buffer B (100% acetonitrile). Identity of peaks is indicated. B, time course for DHHC3 catalyzed palmitoylation of myrGCG (0.5 $\mu$M) with palmCoA (0.5 $\mu$M). Nonlinear regression fit the overall reaction to a single phase exponential, whereas values for 0–6 min were fit to a straight line ($R^2 = 0.99$). C, Michaelis-Menten fit to myrGCG titration with DHHC3 (5 nM) and saturating palmCoA (10 $\mu$M) reacting for 4 min. At maximum velocity, only 2.0% of the palmCoA was consumed. D, palmCoA titration with DHHC9 (5 nM) and saturating myrGCG (10 $\mu$M) reacting for 4 min with a maximal consumption of 2.4% of the myrGCG. E, myrGCG titration with DHHC2 (5 nM) and saturating palmCoA (20 $\mu$M) reacting for 4 min with a maximal consumption of 0.52% of the palmCoA. F, palmCoA titration with DHHC2 (5 nM) and saturating myrGCG (10 $\mu$M) reacting for 4 min with a maximal consumption of 1.7% of the myrGCG. A representative of at least three independent experiments is shown for each.
similar (Table 1) as expected under saturating conditions. The $K_m$ values measured for DHHC2 for myrGCG (Fig. 1E, Table 1) and palmCoA (Fig. 1F, Table 1) were similar to those for DHHC3, suggesting that the enzymes have similar affinities for peptide and palmCoA. The DHHC2 $V_{max}$ value was modestly lower than that of DHHC3. Both substrate concentrations were varied around their $K_m$ values for DHHC3 to generate data for a double reciprocal, Lineweaver-Burk analysis. Unfortunately, these data were not adequate to distinguish a ternary complex from a ping-pong mechanism (24).

Direct Measurement of Acyl Transfer from Acyl-DHHC to Protein Substrate—All DHHC proteins tested to date autoc酰-ate a process hypothesized to result from DHHC proteins using a ping-pong catalytic mechanism for substrate acylation. We directly tested whether DHHC enzyme autoacylation is a transfer intermediate. Because DHHC3 was identified as a PAT for G-protein α-subunits (25), we first confirmed that DHHC3, but not the catalytically inactive mutant DHHS3 (DHHC3 C157S), could palmitoylate N-myristoylated $\alpha_i$ (myrGCG) above background levels. Also, as expected, DHHC3, but not DHHS3, became autoacylated during the assay (Fig. 2A).

Using this enzyme/substrate pair, we tracked the transfer of $[^{3}H]$palmitate from $[^{3}H]$palm-DHHC3 to myrGCG. Palmitoyl-DHHC3 was generated by binding partially purified DHHC3 to FLAG affinity resin and incubating with $[^{3}H]$palmCoA to autoacylate. Unreacted $[^{3}H]$palmCoA was washed out, and $[^{3}H]$palm-DHHC3 was eluted with FLAG peptide. Immuno-blotting and fluorography indicated that elutions 2 through 7 contained $[^{3}H]$palm-DHHC3 (Fig. 2B). The absence of thiol-based reducing agents from the buffers was critical for purification of the acylated enzyme. Elutions were pooled, incubated with nonradiolabeled palmCoA, and either myrGCG or buffer. Aliquots removed at various times were stopped, separated by SDS-PAGE, and analyzed by both fluorography and scintillation counting. Fig. 2, C and E, show that at early time points, most of the $[^{3}H]$palmitate was attached to the DHHC3 but over time, it was lost from the enzyme and accumulated on myrGCG. The rate of transfer of $[^{3}H]$palmitate from DHHC3 inversely paralleled the rate of gain of $[^{3}H]$palmitate on myrG (Fig. 2F). We performed a similar analysis with DHHC2 and its substrate myrLck$_{CT}$ (18, 26). DHHC2 radiolabeled with palmitate was isolated as described above for DHHC3 (supplemental Fig. S2). In the single turnover assay, a similar pattern of loss of radiolabel from the enzyme and gain of palmitate on the substrate was observed both by fluorography (Fig. 2D) and by scintillation counting (Fig. 2F). A slower loss of $[^{3}H]$palmitate from both DHHC proteins was observed when incubated without a protein substrate; this is likely due to hydrolysis of the thioester-attached lipid. Inclusion of an excess of nonradiolabeled palmCoA (1 μM) in the reaction was intended to compete with any residual $[^{3}H]$palmCoA not removed during purification and to drive the reaction to completion. However, radiolabeled palmitate remained associated with DHHC2 and DHHC3 even at extended time courses up to 45 min. Spiking the reaction with additional protein substrate after 10 min did not cause the reaction to proceed further (data not shown).

DHHC Enzyme Autoacylation Acyl-CoA Chain Length Specificity Parallels Substrate Acylation Specificity—If enzyme autoacylation is a transfer intermediate, then the protein substrate should only acylate with acyl-CoAs that can also acylate the DHHC protein. To test this prediction, a competition assay was set up in which a 10-fold excess of nonradiolabeled acyl-CoAs of different chain lengths and saturations was used to compete with $[^{3}H]$palmCoA (C16:0) for acylation of enzyme and substrate. For both DHHC2 and DHHC3, the level of enzyme autoacylation paralleled that of substrate acylation for each competing acyl-CoA tested (Fig. 3, A and B). Together with Fig. 2 and work by others (27), these data strongly suggest that DHHC autoacylation represents a transient transfer intermediate and that DHHC proteins use a two-step ping-pong mechanism for catalysis.

| Table 1 | Kinetic parameters for DHHC3 and DHHC2 analyzed with fluorescent peptide, HPLC-based PAT assay |
|---------|--------------------------------------------------------------------------------------------------|
|         | $K_m$ (μM) | $V_{max}$ (pmol/min/pmol) | $K_m$ (μM) | $V_{max}$ (pmol/min/pmol) |
| DHHC3   | 1.3 ± 0.3 | 11.9 ± 1.1 | 5.9 ± 0.6 | 8.3 ± 1.1 |
| DHHC2   | 0.84 ± 0.07 | 5.2 ± 0.2 | 5.2 ± 1.2 | 11.9 ± 1.1 |

* MyrGCG titrations of DHHC3 was at 10 μM palmCoA, and titration of DHHC2 was at 20 μM palmCoA.
* Palmitoyl-CoA titrations of DHHC3 and DHHC2 were performed at 10 μM myrGCG.
* Values are the means ± S.E. for three independent experiments, except myrGCG titration of DHHC3 which represents five independent experiments.
slower with stearoyl-CoA (Fig. 5B). To determine whether the reduced activity of DHHC3 with stearoyl-CoA was due to more rapid hydrolysis of the acyl group from the enzyme, the production of free fatty acid from the enzyme was monitored from the same reactions analyzed in Fig. 5, A and B. The rate of hydrolysis of stearoyl-DHHC3 was slower than that for palmitoyl-DHHC3 (Table 2), indicating that hydrolysis of the acyl enzyme intermediate did not account for the lower level of autoacylation with stearoyl-CoA. Although the reduction of DHHC3 autoacylation was sizeable, it seemed inadequate to explain the reduction in acylated peptide observed (Fig. 4). Accordingly, we measured the rates of stearoyl transfer to protein substrates using the single turnover assay. DHHC3 displayed a substantially slower rate of acyl transfer to substrate with stearate (Fig. 5C) than with palmitate (Fig. 2D). For DHHC2, the difference in transfer rates for stearate (Fig. 5D) and palmitate (Fig. 2E) was
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FIGURE 3. Acyl-CoA chain length specificity of DHHC enzyme autoacetylation parallels substrate specificity. A, nonradiolabeled acyl-CoAs of the indicated chain length and saturation (10 μM). CoA, or buffer were added to reactions containing [3H]palmCoA (1 μM), DHHC3 (20 nM), and myrG_{H9251} (1 μM). Reactions were incubated at 25 °C for 6 min and processed for quantitation by scintillation spectroscopy. [3H]Palmitate incorporation in the presence of each competitor was normalized to the average of two reactions lacking competitor. For DHHC3 without competitor, the 100% values were 112, 114, and 65 fmol, whereas for myrG_{H9251}, they were 646, 630, and 585 fmol. Mean and S.E. are shown for three independent experiments. B, similar to A, except with DHHC2 (20 nM) and myrLck_{NT} (0.5 μM). For DHHC2 without competitor, the 100% values were 56, 46, and 30 fmol, whereas for myrLck_{NT}, they were 1035, 914, and 886 fmol.

less pronounced. Together these results indicate that DHHC3 has reduced activity for stearoyl-CoA in both steps of the enzyme reaction.

DISCUSSION

In this study, we examined the mechanism DHHC proteins use to catalyze acetyl transfer and their acetyl-CoA specificity. An HPLC, peptide-based PAT assay enabled us to determine kinetic constants for each substrate. Single turnover assays confirmed the hypothesis that DHHC autoacetylation represents the transient acyl enzyme transfer intermediate of a two-step ping-pong mechanism. Acyl-CoA competition assays provided further evidence for this mechanism and revealed an unexpected difference in acyl-CoA preferences between DHHC2 and DHHC3. This difference was shown to result from the reduced ability of DHHC3 to autoacetylate with and transfer acyl chains longer than 16-carbon palmitate.

Kinetic Mechanism of DHHC-mediated Protein Acylation—We and others had previously hypothesized that DHHC proteins use a two-step mechanism based on their ability to autoacetylate (11, 12). Unger mann and co-workers (28) used a “two-step reaction mechanism” to describe the activity of the yeast DHHC protein Pfa3p. However, their two steps referred to the binding of protein substrate by the DHHC and a second event encompassing the entire catalytic reaction. More recently, Mitchell et al. (27) proposed a two-step reaction mechanism in the classical kinetic sense to differentiate steps within the catalytic reaction. Using TLC to monitor free palmitate production, they showed that Erf2p has palmCoA hydrolyase activity that occurs with a rapid autoacetylation step and a slower second step involving the transfer of palmitate to water. The addition of Ras2p, a palmitoylation substrate, increased Erf2p consumption of palmCoA but slowed production of free palmitate, suggesting that the palmitate was now being transferred to Ras2p in the second reaction step. Using single turnover assays, we isolated acylated DHHC protein and confirmed that it can transfer its attached lipid group to a protein substrate. This confirmed that DHHC autoacylation represents a transient transfer intermediate, and thus, supports the hypothesis that DHHC proteins use a two-step transfer mechanism.

To further characterize the mechanism of DHHC-mediated acylation, kinetic constants were determined for each substrate using an HPLC, peptide-based PAT assay. These constants were determined by fitting substrate titration data to the Michaelis-Menten equation. The Michaelis-Menten equation assumes that enzyme and substrate(s) are freely diffusible in solution. However, in our experiments, DHHC proteins were associated with detergent micelles. The substrates, palmitoyl-CoA and the myristoylated peptide, partition between micelles and the aqueous solution. It is unclear whether DHHC proteins acquire their substrates from solution or by horizontal diffusion within a lipid environment. If in our kinetic assays, DHHC proteins acquire substrates by horizontal diffusion, then a rapidly acting enzyme could locally deplete its micelle of unreacted substrate. The apparent $V_{max}$ measured for such an enzyme would reflect the rate of substrate repartitioning into the depleted micelle more than it would reflect the actual catalytic reaction rate. Surface dilution kinetic models account for limited two-dimensional horizontal diffusion and offer an alternative model for fitting the data (29).

In single turnover assays, it is interesting to note that in no case did all the lipid transfer off the DHHC, even at later time points. Additionally, at these later times, a similar amount of enzyme remained labeled regardless of whether the acyl group was transferred to protein substrate or was hydrolyzed. It is unknown whether this reflects enzyme that is inactive with lipid still attached or whether there are secondary acylation sites that are not transferred and are less susceptible to hydrolysis. The stoichiometry of DHHC3 autoacetylation was 0.5 palmitoyl per DHHC (Fig. 5A), similar to a value of 0.65 reported for the yeast DHHC protein Erf2p (27). These data suggest that the stoichiometry of autoacetylation in vitro is one acyl chain per DHHC. The autoacetylation site is predicted to be the conserved cysteine of the DHHC motif. Mutation of this canonical DHHC cysteine blocks autoacylation and transfer, but there is no direct evidence that this is the acylated cysteine. Proteomic analysis of DHHC proteins isolated from cells has revealed that a three-cysteine motif (CCX_{7–13}C/S/T) in the cytoplasmic C-terminal domain of DHHC5, DHHC6, and DHHC8 is palmitoylated (30). This motif is not conserved among DHHC proteins, and it seems unlikely that palmitoylation of this domain represents the acyltransfer intermediate. However, it has been proposed that intramolecular transfer of the acyl group from the catalytic cysteine to the distal palmitoylated cysteines could occur (30). Mapping of the sites of in vitro autoacylation is necessary to resolve this issue.

An alternative possibility to account for the incomplete transfer of palmitate from the enzyme to the substrate in the
single turnover assay is an oligomeric enzyme in which a single site is acylated in each monomer, but not all sites transfer. Evidence for multimerization of DHHC proteins is limited to a report that DHHC3 can be coimmunoprecipitated with itself or with DHHC7 when ectopically expressed in tissue culture (31).

Assessing the quaternary structure of purified DHHC proteins is complicated by the difficulty of accurately sizing protein complexes purified in detergents.

It is likely that the two-step transfer mechanism described here and elsewhere is common to all DHHC proteins. For DHHC2 and DHHC3, and for the yeast Ras PAT Erf2/Erf4 (27), the first step of autoacylation was rapid, suggesting that within cells, DHHC proteins in the presence of acyl-CoAs may persist in the acylated state poised for transfer. Regulation of protein acylation may depend on controlling the association between DHHC proteins and their substrates. Indeed, following activity blockade in neurons, DHHC2 was shown to translocate to postsynaptic densities, colocalize with one of its substrates, postsynaptic scaffolding protein PSD-95, and increase PSD-95 acylation (32). Alternatively, the acylation machinery may be constitutively active, and in that case, deacylation may be a regulated step. To date, no post-translational modification has

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**TABLE 2**

|                   | C16:0 palmitoyl-CoA | C18:0 stearoyl-CoA |
|-------------------|---------------------|--------------------|
| DHHC3 (n = 2)     | 0.027               | 0.008              |
| DHHC2 (n = 2)     | 0.030               | 0.038              |

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**FIGURE 4.** DHHC2 displays broader lipid substrate specificity than DHHC3. DHHC2, DHHC3 (10 nM), or enzyme buffer was incubated with myrGCG (5 μM) and acyl-CoAs (10 μM) of the indicated chain lengths for 10 min at 25 °C. Reactions were stopped, extracted, and processed on an HPLC C4 column. For each reaction, the fluorescence chromatogram of S-acylated myrGCG is shown between 9.5 and 15 min.

**FIGURE 5.** Hydrolysis, enzyme autoacylation, and direct transfer of DHHC2 and DHHC3 with C18 stearate and C16 palmitate. A and B, enzyme autoacylation for DHHC3 (A) or DHHC2 (B) was determined by adding sample buffer to aliquots of the reactions described in Table 2 representing 1 pmol of DHHC and resolving it by SDS-PAGE. The DHHC band was excised and processed for scintillation counting. Analyzing a reaction lacking any DHHC was set as the zero time point, allowing the data to be fit to a one-phase exponential by nonlinear regression. Symbols are: ● DHHC3 with C16- CoA, ▼ DHHC3 with C18-CoA, ■ DHHC2 with C16-CoA; and ▲ DHHC2 with C18-CoA. C and D, single turnover assays with stearoyl-DHHC. C, incubation of [3H]stearoyl-DHHC3 (▼) with myrGCG (●) or [3H]stearoyl-DHHC3 alone (●) for the indicated times and processed for scintillation counting. D, similar to C, incubating [3H]stearoyl-DHHC2 (▲) with myrLckNT (●) or [3H]stearoyl-DHHC2 alone (●) for the indicated times. Data were fit to a single exponential.
DHHC Mechanism and Acyl-CoA Specificity

been described for the regulation of DHHC proteins or deacylating enzymes. Although we are beginning to develop a mechanistic understanding of DHHC proteins, more work is needed to understand their structure and regulation.

Fatty Acyl-CoA Substrate Specificity—Investigating the kinetic mechanism of DHHC proteins led us to determine their acyl-CoA chain length specificity. Surprisingly, a difference in acyl-CoA specificity was observed between DHHC3 and DHHC2. In the competition assay shown in Fig. 3, the reduction of [3H]palmitate incorporation into substrate by nonradio-labeled acyl-CoAs was presumed to be because unlabeled acyl groups were transferred onto the substrate. Indeed, this was likely the case for DHHC2 and all acyl-CoAs tested in Fig. 4 as they could be transferred. However, with DHHC3 and C18- or C20:4-CoA, and to a lesser degree with C18:1, very little acyl group was transferred to the peptide (Fig. 4), yet all three caused about 70% reduction of [3H]palmitate incorporation in the competition assay. Thus, it is likely acyl-CoAs longer than 16 carbons were interacting with DHHC3 in such a way as to slow acylation with palmitate. This was confirmed for DHHC3, in which the extent of autoacylation and the rate of transfer of stearate were slower than with palmCoA. The differential sensitivity of DHHC PAT activity to different acyl-CoAs suggests a possible mode of regulation in cells where changes in the availability of different species of acyl-CoA would impact which DHHC proteins were active, and accordingly, which proteins are modified.

Hydrocarbon rulers for filtering different lipid lengths have been characterized for other enzymes. Protein N-myristoyltransferase shows exquisite specificity for 14-carbon acyl-CoAs. Crystal structures of N-myristoyltransferase bound to substrate analogs reveal that specificity is achieved by measuring the fatty acid between an oxygen hole binding the carbonyl and the floor of the hydrophobic pocket. Furthermore, a hydrophobic groove induces bends in myristate that further restrict acyl chain saturation and branching (33). Fen1p and Sur4p subunits of the very long chain fatty acid synthase complex also display acyl chain length specificity, and similar to DHHC proteins, are polytopic integral membrane enzymes (34). The sizing mechanism results from the distance between a cytoplasmic active site and a lysine residue extending from a transmembrane helix within the lipid bilayer. By shifting the lysine up and down turns of the helix, acyl chain specificity was altered. Although DHHC3 does not contain lysine residues within its predicted transmembrane domains, it does contain charged residues that may behave similarly in restricting acyl chain length. The structural basis for DHHC lipid substrate specificity provides a new area for investigation.

Inhibitors of DHHC proteins will be important tools for studying DHHC biology within cells, as well as having potential as therapeutic agents. An increasing number of DHHC proteins have been linked to different human diseases (9). We and others have attempted to find and characterize DHHC inhibitors with limited success (18). To date, 2-bromopalmitate remains the best DHHC inhibitor available, although it lacks specificity among DHHC proteins as well as other cellular enzymes. Given the two-step mechanism of DHHC catalysis, it might be predicted that inhibitors of the autoacylation step would block acylation by all DHHC proteins. However, data presented here indicate that the autoacylation step displays acyl-CoA specificity. Thus, it is likely that inhibitory compounds can be found that are selective for different DHHC proteins, possibly through acyl-CoA analogs with different fatty acid chains. The success with developing potent and highly selective kinase inhibitors that occupy the ATP binding site encourages efforts to identify small molecules that target DHHC autoacylation.

Acknowledgments—We thank Wendy Greentree for technical support and Robert Deschenes for helpful discussions and comments on the manuscript.

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