Oligomerization of DHHC Protein S-Acyltransferases*

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Background: Oligomerization of DHHC palmitoyltransferases has been suggested.

Results: Self-association of DHHC2 and DHHC3 was detected in intact cells and in vitro. Purified enzymes were found predominately as monomers and dimers, with the monomer displaying higher activity than the dimer.

Conclusion: DHHC proteins may exist in a monomer-oligomer equilibrium, with the monomers having higher enzyme activity.

Significance: Oligomerization may represent a mechanism to regulate DHHC enzyme activity.

The formation of dimers or higher-order oligomers is a property of numerous integral membrane proteins, including ion channels, transporters, and receptors. In this study, we examined whether members of the DHHC-S-acyltransferase family oligomerize in intact cells and in vitro. DHHC-S-acyltransferases are integral membrane proteins that catalyze the addition of palmitate to cysteine residues on proteins at the cytoplasmic face of cell membranes. Bioluminescence resonance energy transfer (BRET) experiments revealed that DHHC2 or DHHC3 (Golgi-specific DHHC zinc finger protein (GODZ)) self-associate when expressed in HEK-293 cells. Homomultimer formation was confirmed by coimmunoprecipitation. Purified DHHC3 resolved predominately as a monomer and dimer on blue native polyacrylamide gels. In intact cells and in vitro, catalytically inactive DHHC proteins displayed a greater propensity to form dimers. BRET signals were higher for the catalytically inactive DHHC2 or DHHC3 than their wild-type counterparts. DHHC3 BRET in cell membranes was decreased by the addition of its lipid substrate palmitoyl-CoA, a treatment that results in autoacetylation of the enzyme. Enzyme activity of a covalently linked DHHC3 dimer was less than that of the monomeric form, suggesting that enzyme activity may be modulated by the oligomerization status of the protein.

Mutations in the genes encoding DHHC9 and DHHC15 cause X-linked intellectual disability in humans. DHHC17 and DHHC3 are huntingtin-interacting proteins that palmitoylate huntingtin and other neuronal substrates. Mice lacking DHHC17 or DHHC3 have behavioral and neuropathological deficits that are similar to those of a mouse model of Huntington’s disease and exhibit reduced palmitoylation of key neuronal substrates, suggesting that altered palmitoylation by DHHC17 may contribute to human Huntington’s disease. Links to schizophrenia (DHHC8), tumorigenesis (DHHC9, 11, and 14), and metastasis (DHHC2) have also been reported. Accordingly, elucidating the mechanism and regulation of the DHHC proteins is important for understanding how palmitoylation manifests in physiology and pathophysiology.

The DHHC cysteine-rich domain is essential for protein acyltransferase (PAT)2 activity (6, 7). The mechanism of palmitate transfer to the protein substrate is through an acylenzyme intermediate that is dependent upon the cysteine of the DHHC motif (8, 9). Mutation of the DHHC cysteine to serine or alanine blocks enzyme activity in vitro and is essential for function in vivo (10). Sequence homology of the DHHC proteins is limited outside the DHHC cysteine-rich domain. Most DHHC proteins have a predicted topology of four transmembrane domains with the N- and C termini exposed to the cytoplasm. A few DHHC proteins have an extended N-terminal domain that contains ankyrin repeats and two additional transmembrane domains, a topology confirmed experimentally for the yeast DHHC protein Akrl (11). The 51-amino acid DHHC cysteine-rich domain begins in the cytoplasmic loop between the second and third transmembrane domains, and its hydrophobic C-terminal region is thought to extend into the membrane.

Evidence suggests that the catalytically inactive mutant in which the DHHC cysteine is mutated acts as a dominant negative when expressed in cultured cells (12–14). However, the mechanism by which the mutation interferes with the function of the wild-type protein is unknown. One hypothesis is that DHHC proteins function as oligomers and that mixed oligomers of the wild-type and mutant enzyme are inactive (13). Support for

2 The abbreviations used are: PAT, protein acyltransferase; RLuc, Renilla luciferase; LUC, luciferase; BRET, bioluminescence resonance energy transfer; TCEP, tris(2-carboxyethyl)phosphine; DTSSP, 3,3′-dithiobis(sulfosuccinimidylpropionate); Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; GPCR, G protein-coupled receptor; 2-BP, 2-bromopalmitate; [3H]palmitoyl-CoA, [9,10-3H]palmitoyl-CoA.

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this argument comes from commounoprecipitation experiments that suggest that DHHC3 (also named GODZ (Golgi-specific DHHC zinc finger protein)) forms homomultimers and heteromultimers with DHHC7 (13).

In this study, we investigated whether DHHC proteins oligomerize in cells and in detergent solution. We used DHHC2 and DHHC3 proteins as examples of the larger DHHC protein family. DHHC2 and DHHC3 are localized in different membrane compartments and have distinct sets of protein substrates (3). These proteins express well in mammalian cells and are biochemically tractable, enabling analysis of purified protein. Our results suggest that DHHC proteins form oligomers in cell membranes and may exist in a monomer-dimer equilibrium in which the active enzyme favors the monomeric state.

**EXPERIMENTAL PROCEDURES**

**Reagents—**Coelenterazine h was purchased from Nanolight. Human α-thrombin was purchased from Hematologic Technologies, Inc. The following antibodies were used: anti-FLAG (catalog no. sc-98220, 1:500) from Santa Cruz Biotechnology, and goat-anti-(catalog no. 632592, 1:1000), anti-DHHC2 (catalog no. ab31837, 1:2000) from Abcam, anti-GFP/YFP from Clontech M2 from Sigma (1:3,000), anti-GODZ (DHHC3) (catalog no. sc-98220, 1:500) from Santa Cruz Biotechnology, and goat-anti-mouse or goat anti-rabbit IgG conjugated to HRP from MP Biomedicals (1:1000). [9,10-3H]palmitate (47.7 Ci/mmol, 105,000 dpm/pmol) was purchased from PerkinElmer Life Sciences. [3H]palm-CoA was synthesized and purified as described (15). A protease inhibitor mixture of leupeptin (5 μg/ml), phenylmethylsulfonylfluoride (0.2 mM), Pepstain A (2 μM), and aprotonin (Sigma, catalog no. A6279, 1:2000) was prepared from stock solutions and used at the final concentrations indicated.

**Cell Culture and Transfection—**HEK-293 cells were cultured in DMEM supplemented with 10% fetal bovine serum at 37 °C in 5% CO₂. HEK-293 cells were transfected with Effectene transfection reagent (Qiagen) according to the instructions of the manufacturer. TriEXTM insect cells were cultured in TriExTM insect cell medium (Novagen) at 27 °C.

**Constructs—**BRET constructs were fusions with humanized Renilla luciferase (RLuc) or YFP. A linker sequence encoding -GGGGS was introduced between the protein of interest and RLuc or YFP. For DHHC3-RLuc and DHHC2-RLuc, murine DHHC3 or human DHHC2 cDNA was subcloned into pRLuc(h)N2 or pRLuc(h)N3 (BRET², BioSignal Packard). For DHHC3-YFP and DHHC2-YFP, the YFP coding sequence was first cloned into pCDNA3.1A(-)-myc-his as a BamHI-HindIII fragment to generate a vector for C-terminal YFP fusion proteins (pML1676). Murine DHHC3 or human DHHC2 cDNA was subcloned into pML1676. To generate constructs for N-terminal YFP fusion proteins, the coding sequence of YFP was subcloned into pCDNA3.1A(-)-myc-his, yielding pML1675. The murine DHHC3 cDNA, the C-terminal domain of human Golgin 84 (amino acids 569–731), or human DHHC2 cDNA was subcloned into pML1675. The plasmids pCDNA3.1-GABA₉R2-YFP, pEAK10 HA-FLAG-CXCR4-hRLuc, and pIREs puro3 FLAG-CXCR4-Venus were gifts from Dr. Michel Bouvier (16). For FLAG-DHHC3 and FLAG-G84, the murine DHHC3 cDNA or C-terminal domain of human Golgin 84 (amino acids 569–731) was subcloned into the pCMV5-FLAG vector. For DHHC3-2GF, the murine DHHC3 cDNA was subcloned into pEFGP-N3 (Clontech). Plasmids for murine myrLckNT, mouse DHHC3-FLAG-His6, DHHS3-FLAG-His6, and DHHC2-FLAG-His6 were described previously (15). The plasmids encoding catalytically inactive DHHC2(C156S) and DHHC3(C157S) were generated by site-directed mutagenesis (QuikChange). To construct a tandem DHHC3 dimer, the DHHC3 coding sequence was amplified with PCR primers to generate an Xhol and BamHI fragment that encodes DHHC3 flanked by a FLAG epitope at the N terminus and a thrombin cleavage site at the C terminus. The fragment was inserted into a plasmid in-frame with a linker sequence (GGGGS) fused to DHHC3. The integrity of all plasmid constructs was confirmed by DNA sequence analysis.

**Bioluminescence Resonance Energy Transfer—**BRET assays were conducted following published protocols (17). HEK-293 cells seeded on 12-well dishes were transfected with 5 ng of luciferase donor plasmid (DHHC3-RLuc, DHHC2-RLuc, DHHS3-RLuc, or DHHS2-RLuc) and increasing amounts of acceptor plasmid (DHHC3-YFP, YFP-DHHC3, YFP-G84, DHHC2-YFP, YFP-DHHC2, GAB₉R2-YFP, DHHS3-YFP, or DHHS2-YFP). Transfections were supplemented with empty vector to normalize total DNA added. For BRET control experiments with CXCR4, 100 ng of CXCR4-RLuc and 0–400 ng of CXCR4-Venus were used. Forty-eight hours after transfection, cells were detached from the plates with PBS-EDTA (5 min at 37 °C), transferred to a microcentrifuge tube, and collected by centrifugation at 300 × g for 5 min at room temperature. Cells were suspended in PBS- CaCl₂/MgCl₂ and 40 μl (~25,000 cells) was dispensed into each well of a white 96-well plate (Costar 3912). After adding 10 μl of 25 μM coelenterazine h solution, the samples in the plate were incubated for 10 min at 37 °C. Emission was detected using a Biotek Gen5 plate reader equipped with the appropriate BRET filter set (460/40 nm and 528/20 nm). The BRET signal was calculated by the ratio of emission at 528 nm to emission at 460 nm minus the BRET in the absence of the acceptor. Luciferase expression was determined after 10 min of adding coelenterazine h. For YFP expression measurements, 40 μl of the cell suspension was distributed into black 96-well plates (Costar 3915), and readings were detected by excitation at 485/20 nm and emission at 528/20 nm. Background fluorescence measured in cells not expressing the acceptor (YFPₑ) was subtracted from fluorescence values measured in cells expressing the increasing amounts of acceptor (YFP) to obtain YFP-YFPₑ values. BRET ratios were then plotted as a function of (YFP-YFPₑ)/LUC (luciferase) values. The data for saturation curves are fit using a nonlinear regression equation with a single binding site (GraphPad Prism) (17). BRET curves with DHHC proteins coexpressed with Golgin84 (Fig. 2A) or GABA₉R2 (C) were fit to a straight line.

For BRET experiments in cell membranes, cells were transfected with 5 ng of donor plasmid (DHHC3-RLuc or DHHS3-RLuc) and 200 ng of acceptor plasmid (DHHC3-YFP or DHHS3-YFP) for CXCR4 control experiment, 100 ng of CXCR4-RLuc and 200 ng of CXCR4-Venus plasmids were used. Cells expressing donor alone were used to calculate the
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BRET background. The cells were collected as described above, suspended in buffer A (20 mM Tris (pH7.4), 1 mM EDTA, protease inhibitors), and broken using a ball bearing cell homogenizer on ice. After centrifugation at 100,000 × g for 30 min, the membrane pellet was suspended in buffer A, and the protein concentration was measured by Bradford assay. Membranes (40 μg) were incubated with different concentrations of palmitoyl-CoA for 10 min at room temperature. The samples were used immediately for the BRET assay as described above.

In Vitro PAT Assay—Recombinant baculoviruses expressing DHHC2 and DHHC3 were described previously. DHHC2-FLAG-His was expressed in insect Sf9 cells and sequentially purified by nickel-nitrilotriacetic acid metal chelate and FLAG affinity chromatography as described (15). An N-terminal fragment encoding the first 226 amino acids of the N-myristoylated protein tyrosine kinase Lck, myrLckNT, was expressed in Escherichia coli with N-myristoyltransferase and purified as C-terminal His-6-tagged proteins using established protocols (15, 18).

For the PAT assay in Fig. 1, HEK-293 cells in 6-well plates were transfected with plasmid DNA (400 ng). After 48 h, the cells were collected, and membrane fractions prepared as above. The membrane pellet was suspended in buffer B (20 mM Tris (pH7.4), 1 mM EDTA, 300 mM sucrose, protease inhibitors), and the protein concentration was measured by Bradford assay. Membranes (22.5 μg in 15 μl) were incubated with 5 μl of myrLckNT (final concentration, 0.5 μM) and 30 μl of reaction mix (250 mM MES, 1.8 μM [3H]palm-CoA) at 25 °C for 10 min. The reactions were stopped by addition of 5× gel loading buffer with 10 mM TCEP and heated for 1 min at 50 °C. Reactions were divided for two SDS-PAGE gels and processed for fluorography or scintillation counting using established methods.

For the PAT assay in Fig. 6, the cross-linked enzyme preparations (plus or minus TCEP) were diluted in 5 μl of enzyme dilution buffer (50 mM MES (pH6.4), 100 mM NaCl, 1% glycerol, 0.1% DDM). Final assay concentrations were 25 nM DHHC2, 1 μM [3H]palm-CoA, and 0.5 μM myrLckNT in a total of 25 μl. After incubation for 10 min at 25 °C, the reactions were stopped, the samples were resolved by SDS-PAGE, and radiolabeled palmitate incorporated into the protein substrate was quantified by scintillation counting.

Coimmunoprecipitation—HEK-293 cells in 6-well plates were transfected with plasmids encoding fusion proteins (400 ng each) for 48 h. The cells were collected and solubilized by adding 500 μl of lysis buffer (50 mM Tris (pH7.4), 150 mM NaCl, 10% glycerol, 1% DDM, protease inhibitors), followed by an incubation on a rotator at 4 °C for 2 h. After ultracentrifugation at 100,000 × g for 30 min at 4 °C, 400 μl of the supernatant was incubated with 20 μl of anti-FLAG resin (equilibrated in lysis buffer containing 0.1% DDM) for 2 h at 4 °C. The resin was washed four times with 1 ml of lysis buffer containing 0.1% DDM. Protein was eluted from the resin by 500 ng/μl FLAG peptide. The purified proteins were incubated with thrombin at a 1:25 ratio at 25 °C. At different time points, the treatment was stopped by gel loading buffer for SDS-PAGE or used immediately for an in vitro PAT assay.

Statistics—Data are expressed as means ± S.D. For comparisons between two groups, Student’s t test (two-tailed) was used. p < 0.05 was considered statistically significant.

RESULTS

DHHC Proteins Oligomerize in Mammalian Cells—To determine whether DHHC proteins exist as dimers or higher-order oligomers in living cells, BRET was used to detect homomeric interactions of DHHC palmitoyltransferases in living cells. The bioluminescent enzyme Renilla luciferase was fused to the C terminus of DHHC2 or DHHC3 (DHHC2-RLuc, DHHC3-RLuc). YFP fusions at the N terminus and at the C terminus were generated. Control fusion proteins included GABA4R2-YFP, a G protein-coupled receptor (GPCR) for GABA, and YFP-Golgin 84, a tail-anchored Golgi structural protein (20). The constructs were designed so that luciferase and YFP were exposed to the cytoplasm. Following transfection in HEK-293 cells, all of the fusion proteins were expressed at the appropriate molecular weights (Fig.
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1, A and B, bottom two panels). PAT activity of the DHHC fusion proteins was confirmed by in vitro assays using an N-terminal fragment of N-myristoylated Lck as a substrate (Fig. 1, A and B, top panels). Catalytically inactive DHHC protein fusions were generated by mutation of the DHHC cysteine to serine and are designated as DHHS2 and DHHS3 (Fig. 1).

BRET saturation curves were generated to determine whether DHHC proteins oligomerize. A constant amount of DHHC3-RLuc plasmid was transfected with increasing amounts of DHHC3-YFP, YFP-DHHC3, or YFP-Golgin 84 plasmid. DHHC3-RLuc displayed saturable BRET signals irrespective of whether the YFP moiety was appended at the N- or C-terminus (Fig. 2A). Because BRET is a measure of proximity, proteins that are colocalized on the same membrane may display a BRET signal because of random collisions, but the signal
is typically smaller than that driven by protein-protein interac-
tion and does not saturate with increasing expression. DHHC3
is localized at the Golgi apparatus. As a control for random
interactions of DHHC3 with other Golgi-localized proteins, we
tested DHHC3-RLuc with the integral membrane protein YFP-
Golgin 84. Consistent with their proximity in Golgi mem-
branes, DHHC3-RLuc and YFP-Golgin 84 coexpression
resulted in a BRET signal. However, the signal was lower than
that seen with DHHC3 pairs and was linear with respect to
increasing ratios of YFP:RLuc expression (Fig. 2 A).

A competition assay was used to confirm the specificity of the
protein interaction suggested by the BRET saturation curves. In
this experiment, increasing amounts of DHHC3 tagged with
the FLAG epitope were coexpressed with DHHC3-RLuc and
DHHC3-YFP. The BRET signal was decreased with increasing
expression of the FLAG-tagged DHHC3 construct, whereas
expression of FLAG-Golgin 84 had a minimal effect (Fig. 2B).

Similar experiments were performed with DHHC2 to deter-
mine whether the propensity to oligomerize was found in other
DHHC proteins. Although DHHC2 and DHHC3 share similar
membrane topologies, their sequence homology is only 21%,
and they are localized in different membrane compartments.
Although DHHC3 is localized in the Golgi, DHHC2 is found in
recycling endosomes and at the plasma membrane. DHHC2-
RLuc displayed saturable BRET interactions with YFP-DHHC2
and DHHC2-YFP (Fig. 2C). For a random collision control, the
GPCR GABABR2-YFP was assayed with DHHC2-RLuc. The
positive BRET signal for DHHC2 and the GABA receptor indi-
cated proximity in the same membrane compartment, but this
BRET pair did not display saturation kinetics (Fig. 2C). Inter-
estingly, the maximal BRET values for both DHHC2 and
DHHC3 were higher with the YFP appended to the C terminus
versus the N terminus, demonstrating that the orientation of
the proteins has an effect on BRET signals (Fig. 2, A and C).

Oligomerization of DHHC Proteins Is Regulated by the Acti-
vation State of the Enzyme—We sought to determine whether
enzyme activity influenced the oligomerization status of
DHHC2 and DHHC3. BRET assays were performed to com-

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**FIGURE 3. Regulation of DHHC protein oligomerization.** A, BRET saturation curves were performed in cells coexpressing wild-type DHHC3-RLuc and DHHC3-YFP (D3, ○) or in cells coexpressing the catalytically inactive DHHS3-RLuc and DHHS3-YFP (S3, □). B, BRET saturation curves were performed in cells coexpressing wild-type DHHC2-RLuc and DHHC2-YFP (D2, ○) or coexpressing the catalytically inactive DHHS2-RLuc and DHHS2-YFP (S2, □). C, BRET saturation curves were performed as in A, with the inclusion of a curve for wild-type DHHC3-RLuc coexpressed with catalytically inactive DHHS3-YFP to detect DHHC3/DHHS3 hetero-oligomers (D3 + S3, ×). The effect of 2-BP was assessed on BRET pairs, wild-type DHHC3 (D), wild-type DHHC2 (E), the GPCR CXCR4 (F), catalytically inactive DHHS3 (G), and catalytically inactive DHHS2 (H). HEK-293 cells were transfected and, 24 h later, treated with or without 100 μM 2-BP. BRET saturation curves were generated ~16 h after treatment with 2-BP (control, ○; 2-BP treatment, □). Data are presented as mean ± S.D. of results of three independent experiments.
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Palmitoyl-CoA inhibits BRET of wild-type but not catalytically inactive DHHC3 in cell membranes. A, detection of DHHC3 oligomerization in vitro. BRET assays were performed on membranes prepared from cells transfected with the BRET pairs DHHC3-RLuc and DHHC3-YFP, DHHC3-RLuc and YFP-Golgin 84, DHHS3-RLuc and DHHS3-YFP, and DHHS3-RLuc and YFP-Golgin 84. Transfections were performed with 5 ng of plasmid DNA for luciferase fusions and 200 ng of plasmid DNA for YFP fusions. B, BRET assays were performed on membranes from cells expressing the wild-type DHHC3 BRET pair (D3, ○) or the catalytically inactive DHHS3 BRET pair (S3, □) following incubation with increasing concentrations of palmitoyl-CoA at 25 °C for 10 min. C, replot of data from B, in which the net BRET signal for wild-type or catalytically inactive DHHC3 are normalized to the value of the samples without palmitoyl-CoA. D, as a control for nonspecific effects of palmitoyl-CoA, membranes from cells transfected with the BRET pair CXCR4-RLuc (100 ng) and CXCR4-Venus (200 ng) were assayed immediately following treatment with or without 50 μM palmitoyl-CoA at 25 °C for 10 min. Data are presented as mean ± S.D. of results of three independent experiments. *, p < 0.05; **, p < 0.01; ***, p < 0.001.

To begin to probe the mechanism that underlies oligomerization of DHHC proteins, we assessed whether multimeric forms of the enzyme could be detected in vitro. Membrane preparations from HEK-293 cells expressing DHHC3-RLuc and DHHC3-YFP were tested in a BRET assay, and those expressing a DHHC3 BRET pair displayed significantly higher signal than membranes from cells expressing DHHC3 with a Golgi-localized protein (Fig. 4A). Consistent with the observations in live cells, the BRET signal for the catalytically inactive mutant was higher than that of the active enzyme. In their reaction cycle, DHHC proteins form a palmitoyl-enzyme intermediate in the first step of the reaction, followed by transfer of the same palmitoyl moiety to the protein substrate. The reaction cycle is dependent upon the cysteine of the DHHC motif. We predicted that addition of palmitoyl-CoA to membranes would promote activation of the enzyme and diminish the BRET signal induced by oligomerization of DHHC3. BRET between DHHC3-RLuc and DHHC3-YFP decreased in a dose-dependent fashion when membranes were incubated with increasing concentrations of palmitoyl-CoA. The BRET signal for catalytically inactive DHHS3 was only diminished slightly (Fig. 4B and C). The highest concentration of palmitoyl-CoA used in these experiments had no effect on CXCR4 BRET (Fig. 4D), excluding a nonspecific effect of palmitoyl-CoA addition to membranes.

We also analyzed oligomerization by coimmunoprecipitation of detergent-solubilized proteins. Wild-type or catalytically inactive DHHC3 tagged with GFP was expressed with FLAG-tagged versions of DHHC3. DHHC3-GFP was found in FLAG immunoprecipitates of FLAG-DHHC3 or FLAG-DHHS3 but not with FLAG-tagged Golgin 84 (Fig. 5). Catalytically inactive DHHS3 was more abundant in the immunoprecipitates than the wild-type enzyme, which is consistent with the BRET studies and supporting the contention that oligomerization favors the inactive state of the enzyme.

As an independent means of assessing the effect of enzyme activity on oligomerization, we used an inhibitor of palmitoylation, 2-bromopalmitate (2-BP). 2-BP inhibits palmitoylation of proteins in cells and DHHC PAT activity in vitro. BRET assays were performed on cells expressing DHHC protein fusions following an overnight treatment with 2-BP. The BRET signal produced by the DHHC3 constructs was modestly but significantly increased by 2-BP treatment compared with the vehicle control (Fig. 3D). A similar effect of 2-BP on DHHC2 oligomerization was observed (Fig. 3E). 2-BP is pleiotropic in its effects. To assess whether the increased BRET signal observed with 2-BP treatment was due to a general perturbation of membranes or protein aggregation, we used as a control, Chemokine (C-X-C motif) receptor 4 (CXCR4), a GPCR that was characterized previously as an oligomer using BRET (16). In contrast to the DHHC proteins, treatment with 2-BP modestly inhibited the CXCR4 BRET signal (Fig. 3F). Thus, the enhancement of the BRET signal observed with 2-BP treatment of DHHC2 and DHHC3 is not common to all BRET pairs. Importantly, 2-BP had no effect on the BRET signal of the catalytically inactive mutants (Fig. 3G and H). Taken together, these results suggest a strong correlation between oligomerization and the inactive enzyme.

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We used blue native gel electrophoresis to assess the oligomeric status of DHHC3 purified from insect cells. In the absence of SDS, both wild-type and catalytically inactive DHHC3 migrated as two bands. When incubated in the presence of SDS, the two forms collapsed into a single band that migrates at a position consistent with the monomeric molecular weight of 34 kDa. The upper band migrates between the 66- and 80-kDa markers, suggesting that it represents a dimer. The dimeric form of the enzyme is more abundant in the catalytically inactive DHHC3 preparation (Fig. 6, A). Purified DHHC3 behaves as a monomer and a dimer on blue native gel electrophoresis. A, purified DHHC3-FLAG-His-6 or DHHS3-FLAG-His-6 were resolved by blue native gel electrophoresis in the presence or absence of SDS as described under “Experimental Procedures.” Transferrin (80 kDa), BSA (66 kDa), and chymotrypsin (25 kDa) were used as molecular weight markers. B, blue native gels were scanned directly, and the ratio of dimer and monomer bands was quantified using ImageJ. Data are presented as mean ± S.D. of three experiments. *

**DISCUSSION**

In this study, we examined whether DHHC proteins self-associate in cells and in vitro. Using BRET, we detected homomultimerization of DHHC2 and DHHC3 in intact cells and in membrane preparations. Evidence that the BRET assays were reporting a protein–protein interaction as opposed to crowding in membrane preparations. Evidence that the BRET assays were reporting a protein–protein interaction as opposed to crowding in membrane preparations was detected by SDS-PAGE and blue native gel electrophoresis. We also used blue native gel electrophoresis to assess the oligomeric status of DHHC3 purified from insect cells. In the absence of SDS, both wild-type and catalytically inactive DHHC3 migrated as two bands. When incubated in the presence of SDS, the two forms collapsed into a single band that migrates at a position consistent with the monomeric molecular weight of 34 kDa. The upper band migrates between the 66- and 80-kDa markers, suggesting that it represents a dimer. The dimeric form of the enzyme is more abundant in the catalytically inactive DHHC3 preparation (Fig. 6, A). Purified DHHC3 behaves as a monomer and a dimer on blue native gel electrophoresis. A, purified DHHC3-FLAG-His-6 or DHHS3-FLAG-His-6 were resolved by blue native gel electrophoresis in the presence or absence of SDS as described under “Experimental Procedures.” Transferrin (80 kDa), BSA (66 kDa), and chymotrypsin (25 kDa) were used as molecular weight markers. B, blue native gels were scanned directly, and the ratio of dimer and monomer bands was quantified using ImageJ. Data are presented as mean ± S.D. of three experiments. *, p < 0.05.

The reduced activity of the cross-linked enzyme could be due to inactivation of the enzyme through means other than restricting dissociation of the monomer. As a second approach to assess PAT activity of the monomer and dimeric forms of the enzyme, a stable dimer was generated by linking two DHHC3 molecules with a thrombin cleavage site. After purification from HEK-293 cells, the proteins were treated with thrombin to release monomeric DHHC3 (Fig. 7, C). PAT activity was monitored over the time course of thrombin cleavage. In the absence of thrombin treatment, dimeric DHHC3 was less active than the monomer. PAT activity increased with time as the monomer was released by thrombin cleavage (Fig. 7, D and E). These results suggest that the PAT activity of DHHC3 is modulated by its oligomeric status.
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DHHC2 and DHHC3 oligomers are dynamic in cell membranes. Although the number of subunits in the different oligomeric states in native membranes is unknown, our data with purified enzyme in detergent solution suggest that monomers and dimers predominate. However, Fang *et al.* (13) immunoprecipitated cross-linked dimers and trimers of DHHC3 from transfected mammalian cells (13). We occasionally observed molecular weight species larger than dimers in our experiments, but their appearance was inconsistent. The amount of dimer detected in detergent solution was modest relative to the monomeric protein (Fig. 6). It is unclear at present whether the proportion of monomer and dimer in detergent solution is representative of their status in cell membranes or whether detergent solubilization promotes dissociation. Future experiments will assess oligomerization of purified DHHC proteins reconstituted into artificial phospholipid bilayers.

Interestingly, a single amino acid change that renders the enzyme catalytically inactive is sufficient to increase oligomerization. We cannot exclude the possibility that a conformational change induced by the change in amino acid is sufficient to promote self-association. However, it seems more likely that posttranslational modification of the enzyme with palmitate plays a role. DHHC proteins are palmitoylated at steady state when expressed ectopically in mammalian cells and yeast (21). Palmitoylation of the catalytically inactive mutant of DHHC3 was essentially absent when expressed in yeast (21) or reduced substantially when expressed in mammalian cells. The number and identity of the palmitoylation sites in DHHC2 and DHHC3 is unknown at present, although palmitoylation is at least partially dependent upon the DHHC cysteine. Palmitoylation of integral membrane proteins increases their affinity for lipid rafts (22), and it is possible that the lipid environment has an impact on the propensity of the enzyme to oligomerize.

It will be important to determine whether our findings of DHHC2 and DHHC3 oligomerization extend to other members of the DHHC protein family. It has been reported that DHHC3 forms homomultimers and heteromultimers with DHHC7 (13). DHHC7 is closely related by sequence to DHHC3, and they display similar subcellular localization and overlapping substrate specificity (3, 13). It was suggested that formation of heteromultimers might account for the dominant negative effects of expressing catalytically inactive DHHC proteins in cells (13). If, as our findings suggest, that the oligomer represents a less active state of the enzyme, then sequestration of a DHHC protein in oligomers with a catalytically inactive protein represents a possible mechanism to explain its dominant inhibitory behavior.

We observed that oligomerization of DHHC2 and DHHC3 was increased when enzyme activity was inhibited chemically or by mutation, whereas addition of the substrate palmitoyl-CoA to cell membranes promoted dissociation of DHHC oligomers. These results suggest that the oligomeric form of the enzyme might be inactive. However, our experiments with a stable DHHC3 dimer demonstrated that a covalently linked dimer is active as an enzyme but less active than the monomeric species. It is possible that the covalently linked dimer may have

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3 C. Gottlieb and M. E. Linder, unpublished data.
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sufficient conformational flexibility to functionally dissociate, leading to a switch from an inactive to active state, but at present we can only conclude that enzyme activity is modulated by the oligomeric state of the DHHC protein.

Our working model of a potential monomer-dimer equilibrium for DHHC proteins has preceded in the behavior of other integral membrane proteins. There is emerging evidence that oligomerization is a dynamic process for some GPCRs (23). Dimerization of GPCRs has been studied extensively, but its functional consequences remain incompletely understood. There is consensus that class C GPCRs form obligate and stable dimers (24). Studies suggest that at least some members of the much larger class A family of GPCRs exist in a monomer-dimer equilibrium (23). A recent single-molecule imaging study demonstrated that the M₁-muscarinic receptor dimers form and dissociate on a time scale of seconds, with about 30% of the receptors existing as dimers at a given time (25). A second example is the EGF receptor, which undergoes ligand-induced dimerization and activation. Dimerization of the EGF receptor has also been observed in the absence of ligand where the receptor is in a monomer-dimer equilibrium (26). The functional consequences of ligand-independent dimerization may be an inhibition of kinase activity (27, 28), but there is also evidence to suggest that dimerization is a prerequisite for EGF binding (29). The striking and intriguing correlation that we observe between the oligomeric state of DHHC proteins and their activity suggests that DHHC PATs may represent another example of protein activity regulated by dynamic self-association.

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