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Multiple Palmitoyltransferases Are Required for Palmitoylation-dependent Regulation of Large Conductance Calcium- and Voltage-activated Potassium Channels*

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Palmitoylation is emerging as an important and dynamic regulator of ion channel function; however, the specificity with which the large family of acyl palmitoyltransferases (zinc finger Asp-His-His-Cys type-containing acyl palmitoyltransferase (DHHCs)) control channel palmitoylation is poorly understood. We have previously demonstrated that the alternatively spliced stress-regulated exon (STREX) variant of the intracellular C-terminal domain of the large conductance calcium- and voltage-activated potassium (BK) channels is palmitoylated and targets the STREX domain to the plasma membrane. Using a combined imaging, biochemical, and functional approach coupled with loss-of-function (small interfering RNA knockdown of endogenous DHHCs) and gain-of-function (overexpression of recombinant DHHCs) assays, we demonstrate that multiple DHHCs control palmitoylation of the C terminus of STREX channels, the association of the STREX domain with the plasma membrane, and functional channel regulation. Cysteine residues 12 and 13 within the STREX insert were the only endogenously palmitoylated residues in the entire C terminus of the STREX channel. Palmitoylation of this dicysteine motif was controlled by DHHCs 3, 5, 7, 9, and 17, although DHHC17 was exclusively palmitoylated and targets the C terminus to the plasma membrane. Using RNA interference, we show that DHHC17 may preferentially target cysteine-rich domains. Finally, our approach may prove useful in elucidating the specificity of DHHC palmitoylation of intracellular domains of other ion channels and transmembrane proteins.

S-Palmitoylation, the reversible addition of 16-carbon saturated palmitic acid to intracellular cysteine residues through a labile thioester linkage (1–5), is emerging as an important dynamic and potent determinant of ion channel function. Palmitoylation controls cell surface expression and regulation of many ligand-gated ion channels, including α-amino-3-hydroxy-5-methyl-4-isoxazole-propionate (6), N-methyl-D-aspartate (7), Kainate (8), P2X7 (9), and γ-aminobutyric acid, type A (10–12). Palmitoylation also controls the function of voltage-gated calcium (13–15), sodium (16), and potassium (17–19) as well as other channels such as AQPA4 (20). For example, in Kv1.1 potassium channels palmitoylation controls voltage dependence (18), and in the stress-regulated exon (STREX)3 splice variant of large conductance calcium- and voltage-activated potassium (BK) channels, palmitoylation determines channel regulation by protein phosphorylation (19). However, although functional insights into ion channel regulation by protein palmitoylation are beginning to emerge, the control of channel palmitoylation is poorly understood, as for other palmitoylated proteins (21).

Protein palmitoylation is controlled by the balance of palmitoyl acyltransferases and palmitoyl thioesterases (1–5). Recently the zinc finger DHHC (Asp-His-His-Cys) type-containing protein family has emerged as a large family of palmitoyl acyltransferases with 23 members in the mouse and human genomes (22, 23). Previous studies have implicated the relatively promiscuous palmitoyltransferase DHHC3 (also known as Golgi-specific DHHC zinc finger protein, GODZ) in palmitoylating some ligand-gated ion channels (6, 7, 10, 12). However, the role of DHHC3 or other DHHCs in controlling palmitoylation of other ion channels, including BK channels, is not known. Furthermore, whether different DHHCs display specificity for palmitoylating individual ion channels has not been examined systematically. Elucidation of such DHHC-substrate relationships would provide significant insight into both the functional role of ion channel palmitoylation and the specificity of DHHCs for diverse target proteins.

We have previously demonstrated that a dicysteine motif (Cys12-Cys13) within the alternatively spliced STREX insert of the intracellular C terminus of BK channels (see Fig. 1) is palmitoylated and targets the C terminus to the plasma membrane in the absence of the transmembrane domains (19). In this manuscript, we have thus asked which DHHCs are responsible for palmitoylation of the STREX domain of the BK channel. We

The abbreviations used are: STREX, stress-regulated exon; BK channel, large conductance calcium- and voltage-activated potassium channel; DHHC, zinc finger Asp-His-His-Cys type-containing acyl palmitoyltransferase; HEK293, human embryonic kidney 293 cell; qRT, quantitative real time; siRNA, small interfering RNA; PKA, protein kinase A; PKAc, catalytic subunit of PKA; DMEM, Dulbecco’s modified Eagle’s medium; PBS, phosphate-buffered saline; HA, hemagglutinin; GFP, green fluorescent protein; YFP, yellow fluorescent protein; CRD, cysteine-rich domain.

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took advantage of the robust endogenous palmitoylation of STREX channels in human embryonic kidney 293 (HEK293) cells as an assay system to systematically exploit both loss and gain of function approaches, through siRNA-mediated knockdown of endogenous DHHCs and overexpression of murine recombinant DHHCs, respectively, to interrogate the role of individual DHHCs in controlling STREX palmitoylation. In initial assays we exploited an imaging screen based on the palmitoylation-dependent plasma membrane localization of the intracellular STREX domain of the BK channel in HEK293 cells (19). Our data represent the first systematic analysis of the contribution of individual DHHCs in controlling palmitoylation of a voltage-gated ion channel. These studies reveal that multiple endogenous DHHCs control palmitoylation of STREX and that DHHC17 has the highest selectivity for the STREX Cys12-Cys13 motif. Furthermore, we demonstrate that the DHHCs that regulate STREX palmitoylation can also assemble as a complex with the channel and determine the regulation of STREX channels by protein kinase A (PKA) phosphorylation.

**EXPERIMENTAL PROCEDURES**

**Channel Constructs**—The generation of full-length, C-terminal, and CRD epitope-tagged constructs of the STREX and ZERO variants of the murine BK channel has been described (19). All mutagenesis was performed using QuikChange mutagenesis (Stratagene) with constructs fully sequenced on (19). All mutagenesis was performed using QuikChange ZERO variants of the murine BK channel has been described

**Cell Culture, Transfection, and RNA Extraction**—HEK293 cells were maintained in DMEM containing 10% fetal calf serum in a humidified atmosphere of 95% air, 5% CO2 at 37 °C. The cells were passaged every 3–7 days using 0.25% trypsin in Hanks’ buffered salt solution containing 0.1% EDTA. For RNA extraction or biochemical studies, the cells were grown in 24- or 6-well plates, respectively. For electrophysiological or imaging assays, the cells were plated on glass coverslips within 6-well plates. Twenty-four hours prior to the experiment, the cells were washed, and medium was replaced with DMEM containing ITS serum replacement (Sigma). The cells were transiently transfected with 40–60% confluence using Lipofectamine 2000 (Invitrogen) or FuGENE-HD (Roche Applied Science). For RNA interference, siRNAs were predesigned and supplied by Qiagen. The knockdown of DHHCs was performed in HEK293 cells by using two siRNAs (10–20 nM of each siRNA) for each gene. siRNA transfection was performed using HiperFect (Qiagen). The knockdown of each DHHC at the mRNA level was analyzed using transfection. The cells were first washed twice with PBS (Invitrogen) and then fixed with ice-cold 4% paraformaldehyde in PBS for 15 min at room temperature. The cells were washed three times with ice-cold PBS and quenched with 50 mM NH4Cl in PBS for 10 min. The cells were washed three times in ice-cold PBS before mounting on microscope slides using Mowiol. The cells were initially analyzed under epifluorescence using an inverted Nikon Eclipse 2000 microscope using a 100× oil objective lens. High power confocal images were acquired on a Zeiss LSM510 laser scanning microscope, using a 63× oil Plan Apochromat (NA = 1.4) objective lens, in multi-tracking mode to minimize channel cross-talk. For each independent cell transfection, three or four coverslips/6-well cluster plate were analyzed for each construct. For each coverslip three to five random fields of view were analyzed to determine the number of transfected cells with plasma membrane localization of the respective fusion protein. The average percentage of transfected cells from each well was then determined for each independent transfection (experiment) and normalized to the corresponding wild type STREX control (membrane expression was typically observed in > 95% of transfected wild type STREX fusion proteins). The majority of experiments were performed blind. In addition, a random subset of cells was also analyzed by quantifying the relative peripheral membrane expression compared with the “intracellular” (cytoplasm + nucleus) expression using ImageJ. The ratio of membrane/intracellular fluorescence was determined and normalized to control treated cells assayed in the same experiment under identical conditions. There was no qualitative difference between these approaches. As such all of the data are expressed as percentages of the respective control means ± S.E. for N independent experiments, where N = minimum total number of cells analyzed across experiments for each construct/treatment.

**Palmitoylation Assays, Pulldowns, and Western Blotting**—CSS-palm prediction was performed using the published CSS-palm v2.0 palmitoylation algorithm (24).

**[3H]Palmitic Acid Incorporation**—HEK293 cells were transiently transfected in 6-well cluster dishes (~3 × 106 cells/well) with the HA-tagged constructs as indicated in the respective figure legends. Forty-eight hours post-transfection, the cells were washed, and 1 ml of fresh DMEM containing 10 mg/ml
RESULTS

Cysteine Residues within STREX Are the Only Endogenously Palmitoylated Residues in the Entire C Terminus of BK Channels—Expression of the entire intracellular C-terminal domain of the STREX splice variant of the murine BK channel as a HA- or GFP epitope-tagged construct (STREX-Cterm; Fig. 1a) in HEK293 cells resulted in robust palmitoylation (Fig. 1b) by endogenous palmitoyltransferases (DHHCs) and expression at the plasma membrane in the absence of the transmembrane domains (Fig. 1, c and d). We have previously demonstrated that the di-cysteine motif at amino acid positions 12 and 13 within the STREX insert is essential for this membrane localization and can be conferred by expression of the cysteine-rich domain (CRD) alone, which includes STREX and the immediately upstream heme-binding domain (19). In accordance with this, mutation of both cysteines to alanine (C12A/C13A) abolished palmitoylation of STREX-Cterm by endogenous DHHCs (Fig. 1b) as well as plasma membrane localization (Fig. 1d). In the C12A/C13A palmitoylation-deficient mutants, the fusion protein was robustly expressed as for wild type (Fig. 1b) but displayed a largely nuclear and/or cytoplasmic cellular distribution (Fig. 1c). Plasma membrane localization of the STREX C terminus, or CRD, was also abolished by preincubation of cells with the palmitoylation inhibitor 2-bromopalmitate (28, 29) (Fig. 1d). Furthermore, expression of the ZERO variant C terminus (ZERO-Cterm) that is identical to STREX-Cterm except with the exclusion of the STREX insert (19, 25, 27), was not palmitoylated by endogenous DHHCs in HEK293 cells and displayed a largely cytoplasmic distribution (Fig. 1, b and c). Taken together, these data demonstrate that the only cysteine residues within the entire C terminus of the STREX variant of the BK channel that are endogenously palmitoylated in HEK293 cells are cysteine residues 12 and 13 within the STREX insert.

Multiple Endogenous DHHCs Control Membrane Expression of the STREX Domain—In an attempt to address which DHHCs regulate palmitoylation of the STREX insert, we first examined the mRNA expression of endogenous DHHCs in our HEK293 cells. Using quantitative real time PCR (qRT-PCR) revealed expression of all 23 human DHHCs at the mRNA level (Fig. 2, a and b). In HEK293 cells DHHC4 mRNA was expressed at the highest level with most other DHHC mRNAs expressed at levels between 5 and 20% of DHHC4.

Based on this mRNA expression profile, we exploited multiple siRNAs against each DHHC isoform to allow us to screen the effect of knocking down DHHC isoforms on membrane localization of the STREX domain. For the majority of DHHCs, we could reliably achieve >80% knockdown (i.e. <20% mRNA remaining) of mRNA as determined by qRT-PCR, using two siRNAs/DHHC (Fig. 1c). In a few cases (e.g. DHHC 11 and 19), even using multiple distinct siRNA combinations, we were unable to achieve mRNA knockdown above 50%. Because antibodies are not available to reliably detect most DHHC isoforms, we were unable to monitor DHHC protein levels. Thus to monitor for knockdown efficiency in this system, we transfected siRNA against GFP to knock down expression of the STREX-Cterm-GFP fusion protein. Under our transfection conditions, typically >75% of all cells express the transfected fusion con-

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fatty acid free bovine serum albumin was added for 30 min at 37 °C. The cells were incubated in DMEM/bovine serum albumin containing 0.5 mCi/ml [3H]palmitic acid (PerkinElmer Life Sciences) for 4 h at 37 °C, and then the medium containing the free label was removed. The cells were lysed in 150 mM NaCl, 50 mM Tris-Cl, 1% Triton X-100, pH 8.0, and centrifuged, and channel fusion proteins were captured using magnetic microbeads coupled to HA/GFP antibody (μMACS™ epitope tag isolation kits; Miltenyi Biotech). After washing columns with 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris-Cl, pH 8.0, followed by washes with 50 mM Tris-Cl, pH 7.5, the captured proteins were eluted in SDS-PAGE sample buffer (50 mM Tris-Cl, pH 6.8, 5 mM dithiothreitol, 1% SDS, 1 mM EDTA, 0.005% bromphenol blue, 10% glycerol) prewarmed to 95 °C. The recovered samples were separated by SDS-PAGE, transferred to nitrocellulose membranes, and probed with either a monoclonal GFP antibody (Clontech; 1:3000) or polyclonal HA antibody (Zymed Laboratories Inc.; 1:1000). A duplicate membrane was dried and stained by guest on August 6, 2013http://www.jbc.org/Downloaded from
In the presence of GFP siRNA, less than 2% of all cells displayed significant GFP expression, indicating that the efficiency of GFP knockdown was $\geq 97\%$.

By exploiting this siRNA screen in conjunction with expression of the CRD-YFP construct (to maximize signal/noise ratio), we first assayed the contribution of individual DHHCs to regulate the plasma membrane expression of the CRD-YFP construct in imaging assays. Individual knockdown of DHHCs 3, 5, 7, 9, and 17, but none of the other DHHCs in which high efficiency knockdown could be achieved resulted in a significant reduction in plasma membrane expression of the CRD-YFP fusion protein in HEK293 cells compared with the control (scrambled siRNA) alone (Fig. 2d), implicating these DHHCs in STREX palmitoylation. However, combinatorial knockdown of these DHHCs did not result in additive effects beyond those seen with the largest decrease in membrane expression of the CRD-YFP fusion protein with siRNA against DHHC9 (data not shown).

Combinatorial knockdown was limited by the reduced efficiency of knockdown using multiple siRNAs and was limited by total siRNA concentrations being toxic to cells. In contrast, inhibition of palmitoyltransferase activity using...
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2-bromopalmitate reduced membrane expression of the C12A/C13A fusion protein to <10% of control (Fig. 1d).

Knockdown of DHHCs 3, 5, 7, 9, and 17 also significantly reduced plasma membrane expression of the STREX-Cterm-GFP fusion protein by ~50% (Fig. 3, a and b) compared with the scrambled siRNA control. To test whether siRNA knockdown of DHHCs 3, 5, 7, 9, and 17 in fact regulated palmitoylation status of the STREX domain, we assayed [3H]palmitate incorporation into the STREX-Cterm construct. Importantly, siRNA knockdown of these DHHCs also significantly reduced but did not completely abolish palmitoylation of the STREX-Cterm fusion protein (Fig. 3, c and d). Similar reductions in both membrane expression and palmitoylation were observed when each individual DHHC was knocked down by siRNA. One possible explanation is that knockdown of any one of these DHHCs results in a compensatory reduction in expression of a common DHHC. For example, does knockdown of DHHC 3, 5, 7, or 9 also give a reduced DHHC17 expression? However, at least at the mRNA level, we saw no significant down-regulation of a common DHHC mRNA upon knocking down individual DHHCs (data not shown). Taken together these data suggest that the endogenous DHHCs 3, 5, 7, 9, and 17 are important determinants of the palmitoylation status of the STREX domain.

**DHHC Overexpression Enhances Membrane Expression of the STREX C Terminus**—If endogenous DHHCs 3, 5, 7, 9, and 17 control palmitoylation of the STREX domain, we hypothesized that overexpression of these DHHCs should enhance plasma membrane localization of the STREX C terminus. We thus co-expressed the STREX-Cterm-GFP construct with HA-tagged murine DHHCs (22) and examined the plasma membrane localization of STREX-Cterm-GFP in HEK-293 cells co-expressing both fusion proteins. To facilitate analysis we co-expressed constructs and imaged expression 24 h after expression. Under these conditions STREX-Cterm-GFP membrane localization is typically ~25% (Fig. 4a) of that seen under normal conditions when imaging is performed 48 h after transfection as in Figs. 1 and 3. Under these conditions, overexpression of DHHCs 3, 5, 7, 9, and 17 significantly increased (more than 2-fold) plasma membrane localization of the STREX-Cterm fusion protein (Fig. 4b). This stimulatory effect was not observed in the presence of the palmitoyltransferase inhibitor 2-bromopalmitate (data not shown).

In contrast, overexpression of DHHCs identified in our siRNA screen because not being involved in endogenous palmitoylation of the STREX domain, including those such as DHHCs 11, 19, and 24 in which siRNA knockdown was <70%, had no significant effect on STREX-Cterm-GFP expression at the plasma membrane (Fig. 4c). Furthermore, co-expression of the catalytically inactive palmitoyltransferase mutants DHHS3 or DHHS7 (22) had no effect on membrane expression, demonstrating that the palmitoyltransferase activity of the DHHCs is required rather than an effect via a possible chaperone function (Fig. 4c). To verify that the effect of DHHC overexpression was dependent upon palmitoylation of the STREX domain itself, we analyzed the effect of DHHCs 3, 5, 7, 9, and 17 on the C12A/C13A mutant of the STREX-Cterm fusion protein that is an absolute requirement for palmitoylation by endogenous DHHCs and expression at the plasma membrane (Fig. 1, b–d). Under these conditions membrane expression of the C12A/C13A fusion alone is almost undetectable (Fig. 4d). Surprisingly, overexpression of DHHCs 3, 5, 7, or 9 also significantly enhanced membrane expression of the C12A/C13A fusion protein to levels that in fact approached that observed upon co-expression with the wild-type STREX-Cterm-GFP (Fig. 4d). In contrast, although there was a small effect of DHHC17 to
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Knockdown of DHHC3, 5, 7, 9, or 17 Prevents PKA-mediated Inhibition of the STREX Channel—To address the functional relevance of STREX domain palmitoylation controlled by DHHC3, 5, 7, 9, and 17, we examined the functional cross-talk between the palmitoylation and PKA-dependent phosphorylation of the STREX channel reported previously (19). Phosphorylation of the STREX domain by PKA leads to channel inhibition; however, this inhibition is conditional on the STREX domain being palmitoylated and associated with the plasma membrane (19).

In cells treated with the scrambled siRNA, application of cAMP to the intracellular face of inside-out patches from HEK293 cells expressing full-length STREX channel resulted in robust inhibition of STREX channel activity that is entirely dependent upon endogenous PKA activity closely associated with the channel (19). As reported previously (19), phosphorylation of the STREX domain by PKA leads to channel inhibition; however, this inhibition is conditional on the STREX domain being palmitoylated and associated with the plasma membrane (19).

Intriguingly, the cysteine residue (Cys16) immediately downstream of cysteines 12 and 13 in the STREX domain is also predicted to be palmitoylated, using the CSS-PALM v2.0 algorithm (24). To address whether Cys16 may be a target for overexpressed DHHCs, we made the triple mutant C12A/C13A/C16A, in which all three cysteines are mutated to alanine. The construct was robustly expressed in HEK293 cells but failed to be located at the plasma membrane as predicted (Fig. 4e). However, co-expression of any DHHC now failed to enhance plasma membrane localization of the C12A/C13A/C16A construct (Fig. 4e). Taken together, these data suggest that DHHC17 is most selective for cysteines 12 and 13 within STREX but that exogenous overexpression of DHHCs 3, 5, 7, or 9 can also regulate palmitoylation status and membrane localization via Cys16.

DHHCs Co-immunoprecipitate with STREX BK Channels—Previous studies have suggested that a number of palmitoylated proteins can assemble with their cognate DHHCs (2, 12, 21, 30). Because multiple DHHCs are able to control the palmitoylation status of the STREX domain, we asked whether DHHCs may be able to assemble in a complex with the full-length BK channel. In these studies full-length STREX channels with a C-terminal GFP fusion were co-expressed with HA-tagged DHHCs in HEK293 cells and subjected to reciprocal co-immunoprecipitation assays. Pull-down using anti-HA antibodies resulted in robust immunoprecipitation of DHHCs 3, 5, 7, 9, and 17. Immunoprecipitates were probed for the GFP tag on the BK channel, revealing co-immunoprecipitation with each DHHC (Fig. 5a). Immunoprecipitation controls including cells expressing STREX-GFP channel alone (Fig. 5, control) or beads alone (not shown) did not result in co-immunoprecipitation. Similar results were observed with the reciprocal pulldown in which channels were immunoprecipitated with anti-GFP and probing for the HA tag on the respective GFP (Fig. 5b), except that under these conditions we could not reliably co-immunoprecipitate DHHC5.

FIGURE 3. DHHCs 3, 5, 7, 9, and 17 control STREX palmitoylation and membrane association. a, representative low power confocal sections of HEK293 cells expressing the STREX-Cterm-GFP fusion protein and transfected with siRNA against the indicated DHHC. The scale bars are 20 μm. b, quantification of STREX-Cterm-GFP membrane localization, expressed as a percentage of the membrane localization with the scrambled siRNA, following the respective DHHC knockdown. The data are the means ± S.E. where N = 4 and n > 350 for each siRNA knockdown. c, fluorograph (upper panel) and corresponding Western blot (lower panel) of HA-tagged C-terminal constructs of the BK channel expressed in HEK293 cells. The cells were labeled with [3H]palmitate (3H-palm) for 4 h, and the constructs were immunoprecipitated using anti-HA magnetic microbeads. d, quantification of STREX-Cterm palmitoylation following siRNA knockdown of DHHCs by siRNA as in c. The data are expressed as percentages of palmitate incorporation in the STREX-Cterm construct in scrambled siRNA-treated cells. The data are the means ± S.E., N = 3–4, *, p < 0.05; **, p < 0.01, compared with respective scrambled control group by analysis of variance with post-hoc Dunnett’s test.

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transfected cells inhibited STREX channel activity by 68 ± 8%, n = 7. In contrast, in cells in which DHHC17 was knocked down by siRNA, no significant change in activity was observed (mean change in activity was 10.8 ± 8.3%, n = 5). Thus knockdown of any of the DHHCs implicated in regulating palmitoylation of the STREX domain also controls the regulation of STREX channels by PKA-mediated phosphorylation.

**DISCUSSION**

Our data provide the first systematic analysis of the role of individual DHHC palmitoyltransferases in the palmitoylation and regulation of a voltage-gated ion channel. We demonstrate that the intracellular alternatively spliced STREX domain of BK channels is endogenously palmitoylated by multiple palmitoyltransferases (DHHCs). Using siRNA knockdown, DHHCs 3, 5, 7, 9, and 17 were all shown to control STREX domain palmitoylation and association of the STREX C terminus with the plasma membrane. Importantly, knockdown of these DHHCs also controlled PKA-dependent inhibition of the STREX BK channel that we have previously shown to be the major functional effect of palmitoylation of STREX in BK channels (19).

Previous analysis of ligand-gated ion channels has revealed an important role for DHHC3 (also known as GODZ) in controlling channel palmitoylation (6, 7, 10, 12, 30). DHHC3 is rather promiscuous in its palmitoylation of target proteins (21, 22, 31), and DHHC3 controlled palmitoylation of STREX. DHHC7, which may heteromultimerize with DHHC3 (10), was also implicated in STREX palmitoylation. In addition DHHC9, DHHC5, and DHHC17 also controlled STREX palmitoylation and function. The regulation of STREX by DHHC17 is particularly intriguing because DHHC17 is also reported to palmitoylate other cysteine-rich proteins including SNAP25 (32), cysteine string protein (33), and huntingtin (34). Overexpression assays suggested that DHHC17 has the highest selectivity for the palmitoylated dicysteine motif of STREX (cysteines 12 and 13 in STREX). Taken together, because Cys12-Cys13 falls within a cysteine-rich domain, as a result of inclusion of the alternatively spliced STREX exon, this may suggest that DHHC17 may preferentially palmitoylate cysteine residues within internal cysteine-rich domains of proteins.

The ability of multiple DHHCs to target the same protein appears to be a general recurring theme in protein palmitoylation. However, it is somewhat surprising that knockdown of any one of these DHHCs (3, 5, 7, 9 or 17) has very similar effects on palmitoylation status, membrane association, and the ability to prevent PKA-mediated inhibition of STREX channels. Indeed, although our attempts to simultaneously knock down all of these DHHCs was unsuccessful, there was no significant addi-
tive effect on multiplexing siRNA knockdown. Multiple distinct mechanisms may be involved in this effect, resulting in each DHHC having an effect in controlling palmitoylation and function, as has been suggested with other proteins. For example, it may simply reflect that the normal cellular expression of each of these DHHCs is required for efficient palmitoylation as the channel traffics to the plasma membrane. It may also reflect potential different localization of specific DHHC substrate interactions occurring within the trafficking pathway. This may be particularly important for tetrameric proteins like BK channels such that a combinatorial code of palmitoylation on multiple sites across multiple subunits is important for the overall palmitoylation status and functional effect. For example, eight cysteine residues (i.e., 4× Cys<sup>12</sup> and Cys<sup>13</sup> in STREX) would be available for palmitoylation in the tetrameric homomeric channel. Although the majority of DHHCs that control STREX palmitoylation are thought to be Golgi/endoplasmic reticulum-localized upon overexpression (23), the localization of endogenous DHHCs and their potential trafficking is poorly understood because of the lack of available antibodies to characterize many of the endogenous DHHC proteins. Additional mechanisms may also exist. As already discussed, heteromultimerization of DHHCs may occur as previously demonstrated using overexpressed DHHC<sub>3</sub> and DHHC<sub>7</sub> (10), or the activity/localization of DHHCs may themselves be controlled by palmitoylation as has been shown for autopalmitoylation of some DHHCs (2). However, the extent to which other DHHCs heteromultimerize, the role of heteromultimerization, and the
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functional effect of DHHC palmitoylation in native systems are largely unknown.

An additional factor that may also be important is the stoichiometry of BK channel palmitoylation that is required for the functional effects of palmitoylation to be manifest. In this regard, we have previously shown that phosphorylation of only a single STREX domain in the channel tetramer is important for functional regulation (25). Whether this is also the case for palmitoylation remains to be determined.

Increasing evidence suggests that DHHCs may assemble with their target substrates. For example, DHHC3 and 17 assemble with SNAP25 (21), whereas DHHC3 has been shown to assemble with their target substrates. For example, DHHC3 and 17 palmitoylation remains to be determined.

Clearly whether the interaction is direct or results from assembly as a much larger macromolecular complex in native systems warrants further investigation. Furthermore, whether cycles of palmitoylation/depalmitoylation are required as the channels traverse different stages in the pathway leading to delivery to the cell surface remains to be explored.

Intriguingly, overexpression of DHHCs that endogenously control STREX palmitoylation (apart from DHHC17) also allowed access of these DHHCs to a cysteine residue (Cys16) immediately downstream of the Cys12–Cys13 site. This implies that Cys16 may also be a target for palmitoylation in some cell lines in which these DHHCs have access to the site, thus extending the potential repertoire by which STREX channels may be regulated by palmitoylation. However, no other cysteine residues within the entire C terminus of the channel were targets for palmitoylation in HEK293 cells because mutation of Cys12–Cys13 completely abolished palmitate incorporation by endogenous DHHCs.

In conclusion, our work reveals that DHHCs 3, 5, 7, 9, and 17 are important determinants of STREX BK channel palmitoylation, STREX domain interaction with the plasma membrane, and functional regulation. Our approach thus represents the first systematic analysis of ion channel palmitoylation by the multi-member DHHC family of palmitoyltransferases. Our strategy employing both loss and gain of function strategies and utilizing fluorescent fusion proteins to screen for the effects of palmitoylation of plasma membrane expression of palmitoylated domains of transmembrane proteins may serve as an approach to further interrogate the specificity and role of DHHCs in controlling ion channel and other plasma membrane transmembrane protein regulation by protein palmitoylation.

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REFERENCES
1. el-Husseini, Ael-D, and Bredt, D. S. (2002) Nat. Rev. Neurosci. 3, 791–802
2. Fukata, Y., and Fukata, M. (2010) Nat. Rev. Neurosci. 11, 161–175
3. Kang, R., Wan, J., Arstikaitis, P., Takahashi, H., Huang, K., Bailey, A. O., Thompson, J. X., Roth, A. F., Drisdel, R. C., Mastro, R., Green, W. N., Yates, J. R., 3rd, Davis, N. G., and El-Husseini, A. (2008) Nature 456, 904–909
4. Linder, M. E., and Deschenes, R. J. (2007) Nat. Rev. Mol. Cell Biol. 8, 74–84
5. Resh, M. D. (2006) Nat. Chem. Biol. 2, 584–590
6. Hayashi, T., Rumbaugh, G., and Huganir, R. L. (2005) Neuron 47, 709–723
7. Hayashi, T., Thomas, G. M., and Huganir, R. L. (2009) Neuron 64, 213–226
8. Pickering, D. S., Taverna, F. A., Salter, M. W., and Hampson, D. R. (1995) Proc. Natl. Acad. Sci. U.S.A. 92, 12090–12094
9. Gonnord, P., Delarasse, C., Auger, R., Benihoud, K., Prigent, M., Cuif, M. H., Lamaze, C., and Kanellopoulos, I. M. (2009) FASEB J. 23, 795–805
10. Fang, C., Deng, L., Keller, C. A., Fukata, M., Fukata, Y., Chen, G., and Lüscher, B. (2006) J. Neurosci. 26, 12758–12768
11. Rathenben, J., Kittler, J. T., and Moss, S. J. (2004) Mol. Cell Neurosci. 26, 251–257
12. Keller, C. A., Yuan, X., Panzanelli, P., Martin, M. L., Allred, M., Sassoé-Pognetto, M., and Lüscher, B. (2004) J. Neurosci. 24, 5881–5891
13. Chan, A. W., Owens, S., Tung, C., and Stanley, E. F. (2007) Cell Calcium 42, 419–425
14. Chen, A. J., Carr, K. M., Shirokov, R. E., Rios, E., and Hosey, M. M. (1996) J. Biol. Chem. 271, 26465–26468
15. Hurley, J. H., Cahill, A. L., Currie, K. P., and Fox, A. P. (2000) Proc. Natl. Acad. Sci. U.S.A. 97, 9293–9298
16. Schmidt, J. W., and Catterall, W. A. (1987) J. Biol. Chem. 262, 13713–13723
17. Jindal, H. K., Folco, E. J., Liu, G. X., and Koren, G. (2008) Am. J. Physiol. 294, H2012–H2021
18. Gubitosi-Klug, R. A., Mancuso, D. J., and Gross, R. W. (2005) Proc. Natl. Acad. Sci. U.S.A. 102, 5964–5968
19. Tian, L., Jeffries, O., McClafferty, H., Molyvadas, A., Rowe, I. C., Saleem, F., Chen, L., Greaves, J., Chamberlain, L. H., Knaus, H. G., Ruth, P., and Shipston, M. J. (2008) Proc. Natl. Acad. Sci. U.S.A. 105, 21006–21101
20. Suzuki, H., Nishikawa, K., Hiroaki, Y., and Fujiyoshi, Y. (2008) Biochem. Biophys. Acta 1778, 1181–1189
21. Huang, K., Sanders, S., Singaraja, R., Orban, P., Cjssouw, T., Arstikaitis, P., Yanai, A., Hayden, M. R., and El-Husseini, A. (2009) FASEB J. 23, 2605–2615
22. Fukata, M., Fukata, Y., Adesnik, H., Nicoll, R. A., and Bredt, D. S. (2004) Neuron 44, 987–996
23. Ohno, Y., Kihara, A., Sano, T., and Igarashi, Y. (2006) Biochem. Biophys. Acta 1761, 474–483
24. Ren, J., Wen, L., Gao, X., Jin, C., Yue, X., and Yao, X. (2008) Protein Eng. Des. Sel. 21, 639–644
25. Tian, L., Coghill, L. S., McClafferty, H., MacDonald, S. H., Antoni, F. A., Ruth, P., Knaus, H. G., and Shipston, M. J. (2004) Proc. Natl. Acad. Sci. U.S.A. 101, 11897–11902
26. Tian, L., Coghill, L. S., MacDonald, S. H., Armstrong, D. L., and Shipston, M. J. (2003) J. Biol. Chem. 278, 8669–8677
27. Tian, L., Duncan, R. R., Hammond, M. S., Coghill, L. S., Wen, H., Rusanova, R., Clark, A. G., Levitan, I. B., and Shipston, M. J. (2001) J. Biol. Chem. 276, 7177–7220
28. Jennings, B. C., Nadolski, M. J., Ling, Y., Baker, M. B., Harrison, M. L., Deschenes, R. J., and Linder, M. E. (2009) J. Lipid Res. 50, 233–242
29. Resh, M. D. (2006) Methods 40, 191–197
30. Uemura, T., Mori, H., and Mishina, M. (2002) Biochem. Biophys. Res. Commun. 296, 492–496
31. Fukata, Y., Iwanaga, T., and Fukata, M. (2006) Methods 40, 177–182
32. Greaves, J., Prescott, G., Fukata, Y., Fukata, M., Salau, C., and Chamberlain, L. (2009) Mol. Biol. Cell 20, 1845–1854
33. Greaves, J., Salau, C., Fukata, Y., Fukata, M., and Chamberlain, L. H. (2008) J. Biol. Chem. 283, 25014–25026
34. Yanai, A., Huang, K., Kang, R., Singaraja, R. R., Arstikaitis, P., Gan, L., Orban, P. C., Mullard, A., Cowan, C. M., Raymond, L. A., Drisdel, R. C., Green, W. N., Ravi Kumar, B., Rubinsztein, D. C., El-Husseini, A., and Hayden, M. R. (2006) Nat. Neurosci. 9, 824–831