Dynamic Palmitoylation Links Cytosol-Membrane Shuttling of Acyl-Protein Thioesterase-1 and Acyl-Protein Thioesterase-2 with that of Proto-Oncogene H-Ras Product and Growth Associated Protein-43

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Running title: Palmitoylation of acyl-protein thioesterase-1 and 2

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Background: The mechanism(s) of cytosol-membrane trafficking of APT1 and APT2 and that of their substrates remains unclear.

Results: Palmitoylation facilitates APT1-APT2 membrane-localization. While APT1 self-catalyzes depalmitoylation and that of H-Ras and APT2; APT2 depalmitoylates GAP-43.

Conclusion: Dynamic palmitoylation links trafficking of APT1 and APT2 with that of their substrates.

Significance: We describe a novel regulatory role of cytosolic thioesterases on their substrates.

SUMMARY

Acyl-protein thioesterase-1 (APT1)2 and APT2 are cytosolic enzymes that catalyze de-palmitoylation of membrane-anchored, palmitoylated H-Ras and growth-associated protein-43 (GAP-43), respectively. However, the mechanism(s) of cytosol-membrane shuttling of APT1 and APT2, required for depalmitoylating their substrates, H-Ras and GAP-43, respectively, remained largely unknown. Here we report that both APT1 and APT2 undergo palmitoylation on Cys-2. Moreover, blocking palmitoylation adversely affects membrane-localization of both APT1 and APT2 and that of their substrates. We also demonstrate that APT1 not only catalyzes its own depalmitoylation but also that of APT2 promoting dynamic palmitoylation (palmitoylation-depalmitoylation) of both thioesterases. Further, shRNA-suppression of APT1 expression or inhibition of its thioesterase activity by palmostatin B markedly increased membrane-localization of APT2 whereas shRNA-suppression of APT2 had virtually no effect on membrane–localization of APT1. In addition, mutagenesis of active site Ser residue to Ala (Ser119Ala), which renders catalytic inactivation of APT1 also increased its membrane localization. Taken together, our findings provide insight into a novel mechanism by which dynamic palmitoylation links cytosol-membrane trafficking of APT1 and APT2 with that of their substrates, facilitating steady-state membrane localization and function of both.

Post-translational modifications such as phosphorylation, glycosylation, ubiquitination and lipidation play critical roles in regulating the function of many proteins (1). Post-translational lipid-modifications of proteins facilitate membrane-localization, protein-protein interaction, cell signaling, subcellular trafficking and vesicular transport (1-8). Some of the common lipid-modifications of proteins include N-myristoylation, palmitoylation and prenylation, which occur in the cytoplasmic face of the cell membrane (1, 5, 6). Among these, palmitoylation (also called S-acylation) is the only reversible lipid-modification in which a 16-carbon fatty acid (predominantly palmitate) is attached to cysteine residues of polypeptides via thioester linkage (4, 6).
Many soluble proteins require this modification for localization to membranes essential for function especially in the central nervous system (4). While palmitoylation is required for membrane-localization and function of these proteins, depalmitoylation is equally critical for recycling or for degradation by lysosomal hydrolases. Thus, dynamic palmitoylation (palmitoylation-depalmitoylation) has emerged as an important mechanism regulating the function of many important proteins including the α-subunit of G-proteins and the product of proto-oncogene, H-Ras (2, 3, 5-10).

In mammals, palmitoylation is catalyzed by a family of 23 enzymes called palmitoyl-acyltransferases (PATs) (7), while depalmitoylation is catalyzed by 4 thioesterases. Two of these thioesterases, acyl-protein thioesterase-1 (APT1) (11) and APT2 (12) are localized predominantly in the cytoplasm, while the other two, palmitoyl-protein thioesterase-1 (PPT1) (13, 14) and PPT2 (15) are lysosomal enzymes (16, 17). The first cytosolic thioesterase to be characterized was APT1, which catalyzes depalmitoylation of the α-subunit of G-proteins and proto-oncogene H-Ras product in vitro (11) as well as Synaptosomal-Associated Protein 23 (SNAP-23) (18). More recently, a second cytosolic thioesterase, APT2, has been reported to depalmitoylate GAP-43 (Growth Associated Protein 43) (12). While both H-Ras and GAP-43 undergo palmitoylation (3, 4) for membrane-localization, these proteins also require depalmitoylation catalyzed by APT1 and APT2, respectively, in order to detach from the membrane and to be recycled. However, until now it remained unclear how APT1 and APT2, being cytosolic enzymes, catalyze depalmitoylation of membrane-anchored, palmitoylated proteins such as H-Ras and GAP-43. Resolution of this question is pivotal in understanding the roles of these proteins in health and disease. We hypothesized that both APT1 and APT2 undergo dynamic palmitoylation for steady-state membrane-localization in order to catalyze depalmitoylation of their membrane-anchored, palmitoylated substrates, H-Ras and GAP-43, respectively.

In the present study, we demonstrate that both APT1 and APT2 are palmitoylated proteins and Cys-2 in both proteins is palmitoylated. We also show that blocking palmitoylation adversely affects membrane-localization of APT1 and APT2 as well as that of their substrates, H-Ras and GAP-43, respectively. Moreover, APT1 not only catalyzes self-depalmitoylation but also that of APT2. This promotes dynamic palmitoylation (palmitoylation-depalmitoylation) of both of these cytosolic thioesterases. Further, we tested shRNA-suppression of APT1 expression and inhibition of its thioesterase activity by an inhibitor, Palm B, which showed elevated levels of membrane localization. In addition, since it has been reported that mutation in any amino acid residue in the active-site triad (GXSXG) causes catalytic inactivation of APT1 (19, 20), we performed mutagenesis studies using (Ser119Ala) in APT1, which markedly increased membrane-localization of both APT1 and APT2 whereas shRNA-suppression of APT2 had virtually no effect on membrane-localization of APT1. Taken together, our results provide insight into a novel mechanism by which dynamic palmitoylation of both APT1 and APT2 facilitates their own cytosol-membrane shuttling as well as that of their substrates. This facilitates steady-state membrane localization and function of APT1 and APT2 as well as H-Ras product and GAP-43, respectively.

**EXPERIMENTAL PROCEDURES**

**Neurosphere culture and astrocyte differentiation in vitro**-All animal experiments were conducted under a protocol approved by the NICHD Animal Care and Use Committee. Mouse neurospheres were isolated from the brain tissues derived from 15-day-old fetuses. The cells were cultured in NeuroCult NSC Basal Medium (Stem-Cell Technologies) containing 10% NeuroCult NSC proliferation supplements and human epidermal growth factor (final concentration of 20ng/ml). To achieve astrocyte differentiation, the proliferating neurospheres were cultured in DMEM media supplemented with 10% FBS. The cultures were incubated at 37°C under an atmosphere of 5% CO₂ and 95% air.

**Mutagenesis of APT1 and APT2-APT1** and APT2 cDNA constructs tagged with Myc-DDK were bought from OriGene Technologies, Inc. (Rockville, MD). To generate palmitoylation site mutants in both APT1 and APT2 mutants, the Cysteine residue in position 2 of the protein sequence (Cys-2) were mutated to Serine. The resulting mutant constructs are designated as follows: Myc-DDK-APT1-mutant (Cys2Ser) as APT1-M-1, Myc-DDK-APT2 mutant (Cys2Ser) as APT2-M-1. Mutagenesis was performed by PCR using the following primers: For APT1-M-1: Forward: 5’-G C C G C G A T C G C C A T G A G C G C A A T A A C A T G T-3’, Reverse:5’-A C A T G T T A T T G C G C C T C A T G G C G A T C G C G C G C-3; For
APT2-M-1: Forward: 5'-C C G C G A T C G C C A T G T C T G G T A A C A C C A C A T G T C-3', Reverse: 5'-G A C A T G G T G T A C C A G A C A T G G C G A T C G C C G-3'. For generating APT1 active site mutant (Ser119Ala), designated APT1-M-2, we used following primers: Forward: 5'-C T T C T A C A G A A T T T T G G G A G G G T T G C T C A G G G A G A G C T G T T A T T C T T A T A C T G-3', Reverse: 5'-C A G T A T A T A A A G C T C C A C C C C A C C C A A A A A T A A T T C T G T T A G A A G-3'. These mutants were generated by PCR using the method of QuickChange site-directed mutagenesis from Agilent Technologies. The mutations were confirmed by DNA sequencing.

**Determination of APT1 and APT2 palmitoylation by ABE method** - Palmitoylation of APT1 and APT2 was assayed by acyl-biotinyl exchange method (ABE) as previously described (21) with minor modifications. Briefly, HEK293T cells were transfected with APT1-cDNA or APT2-cDNA or APT1-M-1 or APT2-M-1 cDNA constructs. The transfected cells were lysed with RIPA buffer (Pierce) and the lysates were incubated overnight with 10 mM N-ethylmaleimide (NEM, Pierce) plus 1 X protease inhibitor (PI) cocktail (Pierce) at 4°C with gentle mixing. NEM was then removed by three sequential precipitations using chloroform-methanol (CM) method as described previously (22). Following third precipitation, the protein precipitate was divided into two equal aliquots. One aliquot was mixed with 1 M hydroxylamine (Sigma) pH 7.4 (freshly prepared), 1 mM HPDP-biotin (Pierce), 0.2% Triton X-100 (Sigma), and 1 x PI and the other aliquot was treated with the identical mixture except that it did not contain hydroxylamine. Both aliquots were then incubated for 1 h at room temperature. The proteins were precipitated by CM-method and treated with 200 µM HPDP-biotin, 0.2% Triton X-100, and 1xPI at room temperature for 1 h. HPDP-biotin was then removed by three sequential CM precipitations. Following the third precipitation, proteins were immunoprecipitated with streptavidin agarose (Pierce) and eluted with SDS-PAGE loading buffer containing 5% β-mercaptoethanol by boiling for 5 min. Samples were then subjected to Western blot analysis with c-Myc antibody (Sigma). Experiments were repeated at least three times.

**Cell fractionation** - Cytosolic and membrane fractions from cultured astroglia were prepared using a previously reported protocol (23) with minor modifications. Briefly, cells were harvested and rinsed with PBS before homogenizing at 4°C followed by a brief centrifugation (500 X g) to pellet the intact cells and nuclei. Supernatants were decanted carefully and centrifuged at 16,000 X g and the membrane and cytosolic fractions were collected. The supernatant
was collected as cytosolic fraction. The pellet was dissolved in 2% triton-X 100 with PI for an hour on ice and centrifuged at 16,000 X g and the supernatant containing the membrane fraction was collected.

*Western blot analyses*: Protein samples (20 μg) were resolved by electrophoresis using 4%–12% SDS-polyacrylamide gels (Invitrogen) under denaturing and reducing conditions. Proteins were then electrotransferred to nitrocellulose membranes (Invitrogen). The membranes were blocked with 5% non-fat dry milk (Bio-Rad) and then subjected to immunoblot analysis using standard methods. The primary antibodies used for the immunoblots were anti-APT1 (Epitomics), anti-APT2 (Novus USA), anti-GAP43 (Chemicon), anti-Myc (Sigma), anti–Pan-Cadherin (Cell signaling) and anti–β-actin (US Biological). The secondary antibodies were goat anti-rabbit IgG (Santa Cruz Biotechnology Inc.) and rabbit anti-mouse IgG (Santa Cruz Biotechnology Inc.). Chemiluminescence was detected using SuperSignal west pico luminol/enhancer solution (Pierce Biotechnology) according to the manufacturer's instructions. Experiments were repeated at least three times and reproducibility confirmed.

*Bromopalmitate- and Palm B-treatment of cultured astroglia*: Astroglia were cultured in DMEM media supplemented with 10% FBS at 37°C under humidified atmosphere containing 5% CO2. Cells were treated with DMSO and varying concentrations of Bromopalmitate (final concentration of 25µM or 50 µM) or Palmostatin B (final concentration of 0.5 µM, 1µM or 2 µM) for 12 hours with change of fresh media containing Palm B every 6 hours. Protein samples were prepared from the treated cells and were used for Western blot analysis. To visualize the subcellular localization of APT1, APT2, GAP43 or H-Ras, treated astrocytes were fixed and analyzed immunocytochemically under confocal microscope.

*shRNA-mediated knock-down of APT1 and APT2 in astroglia*: Cultured astroglia were plated into 75 cm² flasks and 4-chamber slides (Nunc). The following day, each of the constructs. Protein fractions for Western blot analysis were performed as described above.

*Confocal microscopic imaging*: Cultured astrocytes and NIH 3T3 cells were maintained at 37 °C in an atmosphere of 5% of CO2 and 95% air for 72 h on slide chambers (Nunc). The cells were washed 3 times with PBS, pH 7.2, and fixed in 4% paraformaldehyde solution for 15 min at room temperature. The primary antibodies used were anti-APT1 (Epitomics), anti-APT2 (Novus, USA), anti-GAP43 (Chemicon), anti-Myc (Sigma), anti-Na+/K+ATPase (Millipore), anti-Flag (Sigma) and anti-GFP (Abcam). The secondary antibodies used were Alexa Fluor 488–conjugated anti-rabbit, Alexa Fluor 594–conjugated anti-rabbit (Invitrogen), Alexa Fluor 594–conjugated anti-mouse and Alexa Fluor 488-conjugated anti-mouse (Invitrogen). Nuclei were stained with DAPI (Sigma-Aldrich). Fluorescent images of the cells were captured using a Zeiss LSM 510 Inverted Meta confocal microscope or Zeiss Axioskop2 plus fluorescence microscope (Carl Zeiss), and the images were processed using the LSM image software (Carl Zeiss). In each experiment, images were acquired using identical settings and the same standard was applied for all groups. All experiments were repeated at least 3 times.

*Co-localization and quantitation of immunofluorescence*: Quantification of co-localization was performed with Carl Zeiss AIM 4.2 software (Carl Zeiss). The images of overlapping area of immunofluorescence (FITC) from APT1 or APT1M and APT2 or APT2M with those of the membrane marker, Na+/K+ ATPase (Rhodamine fluorescence) were selected manually with PROCESS tab of the software and co-localization of the fluorescence in chosen areas were analyzed with IMAGE CALCULATOR function and Pearson correlations were analyzed by INTENSITY-BASED COLOCALIZATION function of the software.

**RESULTS**

*Cysteine-2 in APT1 and APT2 is Palmitoylated*: To determine whether APT1 and APT2 undergo palmitoylation, we first analyzed the peptide sequences of both mouse- and human-APT1 and APT2 by CSS-Palm (24), a computer program that predicts potential palmitoylation site(s) in polypeptides. Our results showed that Cys-2 of both APT1 and APT2 of mice (Fig. 1A, upper) and humans (Fig. 1A, lower) is a potential palmitoylation site. To delineate whether APT1 and APT2 undergo palmitoylation, we
transfected HEK 293T cells with Myc-Flag (DDK)-tagged-APT1-cDNA- or Myc-Flag (DDK)-tagged-APT2-cDNA-construct (herein after called APT1- or APT2-construct) or Myc-Flag (DDK)-tagged-mutant (Cys 2 Ser)-APT1-cDNA- or Myc-Flag (DDK)-tagged-mutant (Cys 2 Ser) APT2-cDNA-construct (herein after called APT1-M-1 or APT2-M-1 construct) and analyzed the cell lysates for palmitoylated APT1 and APT2 using ABE method (21). The results showed that palmitoylated APT1 (Fig. 1B, lane 2) and APT2 (Fig. 1C, lane 2) are readily detectable in total lysates of HEK 293T cells transfected with either APT1- or APT2-construct, respectively. However, the Palmitoylated APT1- and APT2-protein bands were not detectable in total lysates of cells transfected with APT1-M-1 (Fig. 1B, lane 4) or APT2-M-1 construct (Fig. 1F, lane 4). To further confirm these results, we also labeled the cells transfected with either APT1- or APT2-construct or vector only with [14C]palmitate. The lysates of the labeled cells were immunoprecipitated with Flag-antibody and an aliquot each of the immunoprecipitates was pretreated with hydroxylamine (HA), which cleaves thioester linkage in Palmitoylated proteins (5, 6), before they were resolved by SDS-PAGE and Western blot analysis. The results confirmed that [14C]palmitate was incorporated in both APT1- (Fig. 1D, lane 1) and APT2-protein (Fig. 1E, lane 1) via thioester linkage as the radioactive palmitate label was completely removed by HA-treatment and consequently, no protein band was detectable (Fig. 1D, lane 2 and Fig. 1E, lane 2). As expected, the cells transfected with vector only failed to show radioactive palmitoylated APT1- (Fig. 1D, lane 3) or APT2- (Fig. 1E, lane 3) protein bands. Taken together, these results clearly showed that both APT1 and APT2 undergo S-palmitoylation on Cys-2.

Membrane-localization of APT1 and APT2 requires palmitoylation on Cysteine-2-To determine whether APT1 and APT2 are localized to the membrane and whether the membrane-association depended on Cys-2 palmitoylation, we fractionated HEK 293T cells transfected with either APT1- or APT1-M-1-construct (Fig. 1F) or APT2- or APT2-M-1-construct (Fig. 1G) into cytosolic and membrane fractions. Proteins from these fractions were analyzed by Western blot using Myc-antibody. The results clearly showed that APT1 and APT2 protein bands in the cytosolic fractions of both APT1- and APT1-M-1-transfected (Fig. 1F, lanes 1 and 2) and APT2- and APT2-M-1-transfected (Fig. 1G, lanes 1 and 2) cells were clearly detectable. While the membrane fractions from APT1- (Fig. 1F, lane 3) and APT2-transfected (Fig. 1G, lane 3) cells showed the APT1- and APT2-protein bands, those from APT1-M-1 (Fig. 1F, lane 4) or APT2-M-1 transfected cells failed to show those bands (Fig. 1G, lane 4). These results strongly suggested that Cys-2 palmitoylation is required for membrane-localization of both APT1 and APT2 proteins.

To confirm the membrane-localization of APT1 and APT2, we performed confocal microscopic analysis of cultured mouse astrocytes transfected with either APT1- or APT2-construct or APT1-M-1 or APT2-M-1 construct using Flag- and Na'/K' ATPase antibodies, respectively. We used cultured astrocytes because numerous palmitoylated proteins including the product of H-Ras oncogene and GAP-43 are expressed in the central nervous system (2). The results showed that APT1- (Fig. 1H, upper panels) and APT2- (Fig. 1I, upper panels) but not APT1-M-1- (Fig. 1H, lower panels) and APT2-M-1-transfected (Fig. 1I, lower panels) astrocytes had membrane-localization of APT1 or APT2 protein. Membrane-localization was ascertained by colocalization (merge) of Flag-immunofluorescence with that of Na'/K'ATPase-specific-immunofluorescence. As expected, the tag-immunofluorescence in APT1-M-1 (Fig. 1H, lower panels) and APT2-M-1 transfected (Fig. 1I, lower panels) cells failed to colocalize with Na'/K'ATPase-specific-immunofluorescence. These results strongly suggested that membrane localization of APT1 and APT2 requires palmitoylation on Cys-2.

To further confirm that APT1 and APT2 localized on the cell membrane, we performed quantitative analysis of colocalization of APT1- and APT2-immunofluorescence with that of the membrane marker, Na+/K'ATPase, using Carl Zeiss AIM 4.2 software (Carl Zeiss). Although the results confirmed colocalization of APT1 (Fig. 2A, Panel i) and APT2 (Fig. 2A, Panel ii) with the cell membrane marker, Na+/K+ATPase, there was virtually no colocalization of APT1-M-1 (Fig. 2A, Panel iii) and APT2-M-1 (Fig. 2A, Panel iv) with the cell membrane marker. Pearson’s correlation coefficient (Rr) for APT1 and APT2 (Fig. 2B) was greater than 0.75 in the overlapping areas (mean of 7 individual measurements). Moreover, there was no significant colocalization of APT1-M-1 and APT2-M-1 immunofluorescence with that of the cell membrane marker, Na'/K'ATPase. Taken together, these results strongly suggested colocalization of both the cytosolic
thioesterases on the cell membrane and that colocalization is essential for these enzymes to catalyze self depalmitoylation of APT1 as well as depalmitoylation of APT2 and that of their substrates, H-Ras and GAP-43, respectively.

**Endogenous Apt1 and Apt2 undergoes palmitoylation for membrane localization**—Thus far, we have used overexpression of APT1 and APT2 in HEK 293T cells to experimentally demonstrate that both proteins undergo palmitoylation and that palmitoylation of Cys-2 is required for membrane localization. However, it was not clear whether endogenous APT1 and APT2 (herein after designated as Apt1 and Apt2) require palmitoylation for membrane-association. To determine this, we first treated cultured astrocytes with bromopalmitate, a potent inhibitor of palmitoylation (6), and then resolved the proteins from cytosolic- and membrane-fractions by SDS-PAGE and Western blot analysis using either Apt1- or Apt2-antibody. The results showed that compared with the levels of Apt1 and Apt2 in cytosolic fractions (Fig. 3A and Fig. 3D) those in the membrane fractions gradually declined correlating with increased dose of bromopalmitate (Fig. 3B and Fig. 3E). These results indicated that inhibition of palmitoylation adversely affected membrane-localization of both Apt1 and Apt2 suggesting an essential role of palmitoylation in facilitating translocation of these cytosolic thioesterases to the membrane.

To further confirm that Apt1 and Apt2 require palmitoylation for membrane localization, we first pretreated the astrocytes with bromopalmitate and then performed confocal microscopic analyses of immunofluorescence using antibodies against Apt1, Apt2 and Na⁺/K⁺ATPase. Remarkably, while in DMSO-treated cells (control) colocalization of Apt1 and Apt2 fluorescence with that of Na⁺/K⁺ ATPase was clearly detectable (Fig. 3C, upper panels and Fig. 3F, upper panels), such colocalization was not appreciable in cells treated with bromopalmitate (Fig. 3C, lower panels and Fig. 3F, lower panels). Taken together, these results confirmed that palmitoylation of Apt1 and Apt2 is at least one of the requirements for their membrane-localization.

**Apt1 and Apt2 are dynamically palmitoylated for steady-state membrane localization**—One of the suggested functions of dynamic palmitoylation is to regulate protein sorting (9, 10) and to achieve steady-state membrane localization (2-8, 25, 26). However, recent reports indicate that palmitoylation also regulates protein trafficking to many distinct intracellular compartments due to its sorting role (7, 9). For example, it has been reported that specific PATs and thioesterases regulate surface expression of important proteins such as calcium-activated potassium channels (27). Moreover, palmitoylation increases the affinity for membrane-localization, depalmitoylation plays a vital role in recycling and/or degradation of proteins that undergo S-palmitoylation (9, 10). For example, dynamic palmitoylation regulates recycling of proteins such as the α-subunit of G-proteins (11), the products of H- and N-Ras proto-oncogenes (13) as well as endothelial nitric oxide synthase (28). Recently it has been reported that dynamic palmitoylation regulates T cell activation and anergy (29). Although the specific PATs that catalyze palmitoylation of APT1 and APT2 are yet to be identified, we speculated that APT1 and APT2 may catalyze their own depalmitoylation or they catalyze depalmitoylation of each other in order to promote their dynamic palmitoylation promoting steady-state membrane localization and function. Accordingly, we performed Western blot analysis of cytosolic and membrane fractions of cultured astrocytes that were treated with either DMSO (control) or with varying doses of Palmostatin-B (Palmo-B), first reported to be a catalytic inhibitor of APT1 (30) but subsequently, found to inhibit both APT1 and APT2 (31). The results suggested that while Apt1 (Fig. 4A, lane 1) and Apt2 (Fig. 4B, lane 1) levels in the cytosolic fraction of the cells treated with DMSO alone (control) were virtually identical to those in the cytosolic fractions of Palmo-B-treated cells (Figs. 4A and 4B lanes 2 and 3), the membrane fractions of these cells showed a dose-dependent elevation of Apt1 (Fig. 4A, lanes 5 and 6) and Apt2 (Fig. 4B, lanes 5 and 6) protein levels. These results suggested that inhibition of thioesterase activity markedly increased membrane-localization of both Apt1 and Apt2 raising the possibilities that these two thioesterases may catalyze their own depalmitoylation or that of each other or both.

**Depalmitoylation of palmitoylated APT1 and APT2**—To confirm these results, we transfected HEK293T cells with APT1- or APT2-construct and treated these cells with DMSO (control), bromopalmitate or Palmo-B and determined the levels of palmitoylated APT1 (Palm-APT1) and Palm-APT2 in these cells by ABE method (21). The results showed that compared with the membrane fractions of DMSO-treated cells (Fig. 4C, lane 1 and Fig. 4D, lane 1), bromopalmitate-treated cells had lower levels of both
Palm-APT1 (Fig. 4C, lane 2) and Palm-APT2 (Fig. 4D, lane 2). However, in cells treated with thioesterase inhibitor, Palmo-B, the levels of both Palm-APT1 (Fig. 4C, lane 3) and Palm-APT2 (Fig. 4D, lane 3) were much higher. These results suggested that inhibition of palmitoylation suppresses membrane-localization of both APT1 and APT2 and that inhibition of thioesterase activity leads to higher levels of membrane-associated APT1 and APT2. Next, we immune-histochemically analyzed the Palmo-B-treated cells for cellular distribution of endogenous Apt1 and Apt2. The results showed that compared with the DMSO-treated cells (Fig. 4E and Fig. 4F, upper panels), Palmo-B-treatment markedly increased both Apt1- (Fig 4E, lower panels) and Apt2-immunofluorescence (Fig. 4F, lower panels), which merged with that of the membrane-marker, Na+/K+ ATPase. To confirm these results, we also performed the same experiments using pancadherin as an alternative membrane marker and the results confirmed the findings obtained with Na+/K+ ATPase (data not shown). These results suggested that Apt1 and Apt2 undergo palmitoylation and that either these enzymes self-catalyze depalmitoylation or depalmitoylate each other or both.

Mechanism of dynamic palmitoylation of APT1 and APT2—We next sought to determine which of the above possible mechanisms may actually regulate dynamic palmitoylation and steady-state membrane localization of Apt1 and Apt2. However, since Palmo-B inhibits both Apt1 and Apt2 (30, 31), generalized inhibition of these cytosolic thioesterases may not clearly answer our question. Therefore, we first performed experiments in which cultured astrocytes were transfected with either scrambled-shRNA (control) or APT1-shRNA and determined the levels of Apt1 expression. The results showed that compared with the control (Fig. 5A, lane 1) the APT1-shRNA-transfected cells had a marked inhibition of Apt1 expression (Fig. 5A, lane 2). We then fractionated the control and APT1-shRNA-transfected cells and determined the levels of Apt2-protein levels in the cytosolic and membrane fractions by Western blot analysis. The results showed that compared with the cytosolic fractions of the control cells (Fig. 5B, lane 1) those of the APT1-shRNA-treated cells had a lower level of Apt2-protein (Fig. 5B, lane 2). Interestingly, with the level of Apt2 in the membrane fraction of scrambled-shRNA transfected cells (Fig. 5C, lane 1) that of APT1-shRNA transfected cells showed markedly higher level of Apt2 (Fig. 5C, lane 2). These results indicated that Apt1 catalyzes depalmitoylation of Apt2 promoting its dynamic palmitoylation critical for steady-state membrane localization.

We then performed similar experiments in which the cells were transfected with either scrambled shRNA (control) or APT2-shRNA and determined the level of Apt1-protein in the cytosolic and membrane fractions by Western blot analysis. First we checked the effectiveness of the APT2-shRNA in suppressing Apt2 expression and the results showed that compared with the scrambled shRNA-transfected cells (control) (Fig. 5D, lane 1) those transfected with APT2-shRNA had a marked suppression of Apt2 expression (Fig. 5D, lane 2). We then checked the Apt1-protein levels in the cytosolic and membrane fractions of control and APT2-shRNA-transfected cells by Western blot analysis. The results showed that the levels of Apt1-protein in the cytosolic fractions of control (Fig. 5E, lane 1) and APT2-shRNA-transfected cells (Fig. 5E, lane 2) were virtually identical. Moreover, compared with the membrane fractions of the control cells (Fig. 5F, lane 1) those of the APT2-shRNA-transfected cells (Fig. 5F, lane 2) appeared to contain virtually identical level of membrane-associated Apt1. To confirm these results, we performed confocal microscopic analysis of the cells transfected with scrambled-shRNA (control) or with either APT2- or APT1-shRNA for membrane-localization of Apt1- or Apt2-immunofluorescence, respectively. Colocalization of Apt1- and Apt2-immunofluorescence with that of Na+/K+ ATPase was considered evidence of membrane-localization. The results showed that compared with the level of membrane-localized Apt2–immunofluorescence in control cells (Fig. 5G, upper panels), that of APT1-shRNA-transfected cells (Fig. 5G, lower panels) were markedly higher. However, compared with the control cells (Fig. 5H, upper panels) the APT2-shRNA-transfected cells (Fig. 5H, lower panels) showed virtually identical intensity of membrane-associated Apt1-immunofluorescence. Taken together, these results demonstrated that while Apt1 depalmitoylates Apt2 regulating its steady-state membrane-localization, Apt2 does not regulate that of Apt1.

Dynamic palmitoylation of APT1 and APT2 promotes that of H-Ras and GAP-43, respectively—To determine whether inhibition of thioesterase activity of Apt1 and Apt2 affected membrane localization of H-Ras and GAP-43, respectively, we treated cultured
astrocytes with varying doses of Palmo-B and performed Western blot analysis of cytosolic and membrane fractions of the cells using antibodies to either H-Ras or GAP-43. The results showed that while treatment of the cells with DMSO alone or with 0.5 µM Palmo-B did not appreciably alter the levels of cytosolic H-Ras (Fig. 6A, lanes 1 and 2) and GAP-43 (Fig. 6B, lanes 1 and 2) proteins, treatment with 1µM Palmo-B resulted in slightly lower level of H-Ras (Fig. 6A, lane 3) while the level of GAP-43 remained virtually unaltered (Fig. 6B, lane 3). In contrast, compared with the levels of membrane-associated H-Ras (Fig. 6C, lane 1) and GAP-43 (Fig. 6D, lane 1) in DMSO-treated cells, the membrane fractions of the cells treated with 1µM Palmo-B contained appreciably higher levels of H-Ras (Fig. 6C, lane 3), as well as GAP-43 (Fig. 6D, lane 3). These results provided strong evidence that inhibition of either Apt1 or Apt2 enzymatic activity by Palmo-B impaired depalmitoylation of palmitoylated H-Ras and GAP-43, respectively, and as a result these palmitoylated proteins remained anchored to the membrane.

To further confirm the membrane-localization of H-Ras and GAP-43, we performed experiments identical to those described above and determined colocalization of H-Ras and GAP-43 immunofluorescence with that of the membrane marker, Na+/K+ ATPase using confocal microscopy. The results showed that while in DMSO-treated (control) cells a very low level of GFP-H-Ras (control) cells a very low level of GFP-H-Ras (Fig. 6A, lanes 1 and 2) and GAP-43 (Fig. 6F, upper panels) immunofluorescence co-localized with that of Na+/K+ ATPase using confocal microscopy. The results showed that while in DMSO-treated (control) cells a very low level of GFP-H-Ras (control) cells a very low level of GFP-H-Ras (Fig. 6A, lanes 1 and 2) and GAP-43 (Fig. 6F, upper panels) immunofluorescence co-localized with that of Na+/K+ ATPase (control) cells a very low level of GFP-H-Ras (control) cells a very low level of GFP-H-Ras (Fig. 6A, lanes 1 and 2) and GAP-43 (Fig. 6F, upper panels) immunofluorescence co-localized with that of Na+/K+ ATPase. The results showed that in cells transfected with APT1-construct, APT1-immunofluorescence colocalized with that of the membrane marker (Fig. 7A, upper row) although some fluorescence was also present in the cytoplasm. However, in the cells transfected with APT1-M-1 construct the APT1-immunofluorescence predominately localized in the cytoplasm (Fig. 7A, middle row) and in those cells transfected with APT1 active site (Ser119Ala) mutant, APT1-M-2, high intensity APT1-immunofluorescence colocalized predominantly with the membrane marker, Na+/K+ ATPase (Fig. 7A, lower row). These results strongly suggested that catalytically inactive APT1 preferentially localized to the membrane suggesting that depalmitoylation of APT1 is self catalyzed and that self-depalmitoylation promotes dynamic palmitoylation of APT1, which maintains steady state membrane localization of this thioesterase.

In order to further confirm that self-catalyzed depalmitoylation of APT1 is required for steady-state membrane localization of APT2, we transfected NIH3T3 cells with each of the three constructs described above and analyzed the cells for membrane localization of APT2-immunofluorescence. Our results showed that when the cells were transfected with APT1-construct the APT2-immunofluorescence was colocalized with the membrane marker as well as detectable in the cytoplasm (Fig. 7B, upper row). However, in APT1-M-1-transfected cells APT2-immunofluorescence was localized mostly on the membrane but some also appeared to be localized in the cytoplasm (Fig. 7B, middle row). Remarkably, the cells transfected with Apt1 active-site mutation (Ser119Ala) showed that intense APT2-immunofluorescence colocalized predominantly with that of the membrane marker, Na+/K+ ATPase (Fig. 7B, lower row). These results clearly showed that APT1 catalyzed depalmitoylation of APT2 promoting dynamic palmitoylation of APT2.
DISCUSSION

Several years ago, it was demonstrated that APT1, the first cytosolic thioesterase to be characterized, catalyzed depalmitoylation of the α-subunits of G-proteins in vitro (11). Moreover, APT1 has also been reported to catalyze depalmitoylation of proto-oncogene H-Ras product in cellulo (30). Recently, it has been reported that while endogenous and overexpressed human-APT1 (hAPT1) were predominantly localized to the cytosol, APT1 signals were also detectable on the plasma membrane, the nuclear membrane and in the ER in HEK293 cells (32). However, it remained unclear how cytosolic thioesterases, Apt1 and Apt2, could catalyze depalmitoylation of their palmitoylated substrates like H-Ras and GAP-43 that are anchored to the cell membrane. Interestingly, a recent study of a proteome scale characterization of human palmitoylated proteins in lipid raft-enriched membranes suggested that APT1 is a palmitoylated protein and thus, it is likely to be targeted to the membranes (33). Our results are consistent with this notion and for the first time, provide experimental evidence that both APT1 and APT2 undergo palmitoylation on N-terminal Cys-2. Moreover, our results show that palmitoylation on Cys-2 facilitates membrane-localization of these cytosolic thioesterases. Further, we demonstrated for the first time that Apt1 not only catalyzed its own depalmitoylation but also that of Apt2. Thus, dynamic palmitoylation of Apt1 mediated also facilitated that of Apt2. More importantly, dynamic palmitoylation of Apt1 and Apt2 promoted that of their substrates, H-Ras and GAP-43, respectively. Interestingly, it has been reported that surface expression of calcium-activated potassium channels are regulated by two of the 23 mammalian PATs (DHHC22 and DHHC23), which catalyze palmitoylation, while depalmitoylation is catalyzed only by APT1 and not by APT2 (27). We propose that both of these cytosolic thioesterases require dynamic palmitoylation for their own steady-state membrane localization, which is essential for their function in promoting dynamic palmitoylation and function of their substrates, H-Ras and GAP-43, respectively.

On the basis of these results, we propose a model (Fig. 8) for the role of dynamic palmitoylation of APT1 and APT2 in their steady-state membrane localization and function in regulating those of their substrates, H-Ras and GAP-43, respectively. In this model, cytosolic thioesterases, APT1 and APT2, and their respective substrates undergo palmitoylation in the Golgi by as yet unknown PATs, which facilitates their membrane localization. Membrane-associated APT1 and APT2 then depalmitoylate H-Ras and GAP-43, respectively, detaching them from the cell membrane, which promotes translocation to the Golgi where they are re-palmitoylated and translocated to the membrane, essential for their function. APT1 also depalmitoylates APT2 detaching it from the membrane for another round of palmitoylation. Following depalmitoylation of APT2, APT1 then catalyzes its own depalmitoylation for translocation to the Golgi in order to undergo re-palmitoylation, required for membrane localization. However, while APT1 catalyzes depalmitoylation of APT2, APT2 does not depalmitoylate APT1. Taken together, based upon our findings this model provides insight into a novel mechanism in which dynamic palmitoylation links cytosol-membrane translocation of the two cytosolic thioesterases with that of their substrates, H-Ras and GAP-43.
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FOOTNOTES
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1Equally contributed
2The abbreviations used-APT1, Acyl-protein thioesterase-1; APT2, Acyl-protein thioesterase-2; Ala, Alanine; GAP-43, growth-associated protein-43; SNAP-23, Synaptosomal-Associated Protein 23;PPT1, Palmitoyl-protein thioesterase-1; PPT2, Palmitoyl-protein thioesterase-2; Palm B, Palmostatin B; Ser, Serine.

FIGURE LEGENDS
Figure 1. Palmitoylation of APT1 or APT2 promotes their membrane-localization. Palmitoylation site (Cys-2) was predicted by CSS-Palm 3.0 analysis at the highest stringency in mouse-APT1 and APT2 (panel A, upper two rows) and human APT1 and APT2 (panel A, lower two rows). Palmitoylated-APT1 (panel B, lane 2) and APT2 (panel C, lane 2) were readily detectable by ABE assay, while Cys2Ser mutation in both APT1 (panel B, lane 4) and APT2 (panel C, lane 4) abrogated palmitoylation. Palmitoylation of APT1 (panel D, lane 1) and APT2 (panel E, lane 1) were further confirmed by the presence of [14C]palmitate-labeled APT1- and APT2-protein bands, which were rendered undetectable by hydroxylamine treatment (panels D and E, lanes 2). Note that Cys2Ser mutation in APT1 (panel F) and APT2 (panel G) abrogated membrane-association of both APT1 (panel F, lane 4), and APT2 (panel G, lane 4). Subcellular localizations of APT1 and APT2 in cells transfected with APT1 or APT1-M-1 or APT2 or APT2-M-1 construct were analyzed by confocal microscopy. Image data show that while APT1 (panel H, upper row) and APT2 (panel I, upper row) were localized to the membrane (arrows), the APT1-M-1 (panel H, lower row) and APT2-M-1 (panel I, lower row) were predominantly localized to the cytoplasm.

Figure 2. Colocalization of APT1- and APT2-immunofluorescence with that of Na+/K+ATPase. Quantitation of APT1- or APT2-immunofluorescence with Na+/K+ATPase were performed using Carl Zeiss AIM 4.2 Software (Carl Zeiss) to identify colocalization in the overlapping areas of the image. Insets show colocalization of APT1 (panel A, i) and APT2 (panel A, ii) with cell membrane marker, Na+/K+ ATPase. There were no colocalization of APT1-M-1 (panel A, iii) and APT2-M-1 (panel A, iv) with the cell membrane marker. Pearson’s correlation coefficient (Rr) of APT1 or APT2 is greater than 0.75 in the overlapping areas (mean from 7 individual measurements) (panel B). Moreover, there were no significant correlation of APT1-M-1 and APT2-M-1 with cell membrane marker, Na+/K+ ATPase.

Figure 3. Suppression of Apt1 and Apt2 palmitoylation by bromopalmitate. Suppression of Apt1-palmitoylation by bromopalmitate dose-dependently increased the level of cytosolic Apt1 (panel A) and reduced its membrane-localization (panel B). Immunocytochemical analyses were performed using Apt1- and Na+/K+ ATPase-antibodies, which showed that Apt1 is localized predominantly in the cytoplasm of the cells treated with bromopalmitate (panel C, lower row), while accumulation of Apt1 is clearly detected on the membrane of the cells treated with DMSO (panel C, upper row). The cytosolic and membrane fractions from bromopalmitate-treated cells were probed with Apt2-antibody. Suppression of Apt2-palmitoylation by bromopalmitate markedly increased the cytosolic Apt2 (panel D) and decreased its membrane-localization (panel E). Immunocytochemical analyses were performed using Apt2- or Na+/K+ ATPase-antibodies. Note that Apt2 signal on the cell membrane is virtually abolished in Bromopalmitate-treated cells (panel F, lower row) compared with DMSO-treated control cells (panel F, upper row).
Figure 4. Membrane-localization of APT1 and APT2 in Palm B-treated cells. Palmostatin B (Palm-B)-treatment in a dose-dependent manner elevated the levels of both Apt1 (panel A) and Apt2 (panel B) in membrane fractions of the cells. Palmitoylation status of APT1 (panel C) and APT2 (panel D) were checked in cultured astroglial cells expressing APT1 or APT2 in which either palmitoylation or depalmitoylation was inhibited by bromopalmitate- and Palm B (Palm-B)-treatment, respectively. Lysates from 3 x 10^6 cells for each treatment were used for Western blot analysis. The densitometric quantitation of the protein bands in Western blots from three independent experiments were performed and the results are presented graphically as the mean ±SD. Note that Palm-B treatment markedly elevated the levels of palmitoylated APT1 (panel C, lane 3, and bar graph) as well as APT2 (panel D, lane 3, and bar graph) as compared with their respective controls (panels C and D lane 1), while bromopalmitate-treatment markedly reduced the level of palmitoylated APT1 (panel C, lane 2) and APT2 (panel D, lane 2). Palmo-B treatment prevents dissociation of both APT1 (panel E) and APT2 (panel F) from the membrane. Compared with DMSO-treated cells (panel E and panel F, upper panels, arrows), the Palmo-B-treated cells had markedly elevated levels of membrane-localized Apt1- (panel E, lower panel, arrows) and Apt2-fluorescence (panel F, lower panel, arrows).

Figure 5. Effects of shRNA-suppression of Apt1 or Apt2 on membrane-localization of Apt1 and Apt2. Compared with scrambled shRNA transfected cells (panel A, lane 1) those transfected with APT1-shRNA (panel A, lane 2) had appreciably decreased level of Apt1-protein. Apt1-knockdown reduced the level of Apt2 in the cytosolic fractions (panel B) but increased that of Apt2 in the membrane fractions (panel C). APT2-shRNA-transfection markedly decreased Apt2 expression (panel D, lane 2) compared with that of scrambled-shRNA-transfected cells (panel D, lane 1). Western blot analysis of cytosolic (panel E) and membrane fractions (panel F) from APT2-shRNA transfected cells showed virtually no alteration in Apt1 protein level. Immunocytochemical analysis was performed with either Apt1- or Apt2- and Na^+/K^+ATPase-antibody. Compared with scrambled shRNA-transfected cells (panel G, upper row), those transfected with APT1-shRNA had markedly higher Apt1 signal colocalized with that of Na^+/K^+ATPase (panel G, lower row). However, compared with its scrambled shRNA-transfected cells (panel H, upper panel) those transfected with APT2-shRNA had very similar Apt1 signal colocalization with that of the membrane marker, Na^+/K^+ATPase (panel H, lower row).

Figure 6. Dynamic palmitoylation of Apt1 and Apt2 regulates steady-state membrane localization of H-Ras and GAP-43, respectively. The cytosolic fractions of cultured astroglia treated with DMSO or Palm-B were probed with H-Ras- or GAP-43-antibodies as indicated. Note a slight reduction in H-Ras signal in the cytosolic fraction of 1 μM Palm-B-treated cells (panel A). There is, however, no apparent difference in the levels of GAP-43 (panel B) in the cytosolic fractions of Palm-B-treated and control cells. The membrane fractions from astroglial cells treated with DMSO (control) or varying concentrations of Palm-B were probed with either H-Ras- or GAP-43-antibodies. Note that both H-Ras (panel C) and GAP-43 (panel D) levels in the membrane fractions of the cells treated with Palm-B were elevated in a dose-dependent manner. Immunocytochemical analysis of cultured astroglia were performed using antibodies to either H-Ras (panel E) or GAP-43 (panel F) and Na^+/K^+ATPase. Compared with DMSO-treated cells (panel E and panel F, upper row, arrows), those treated with Palm-B showed markedly increased membrane localization of H-Ras (panel E, lower row, arrows) as well as that of GAP-43 (panel F, lower row, arrows).

Figure 7. Active site mutation of APT1 increases membrane localization of both APT1 and APT2. Cultured astroglia were transfected with APT1-, APT1-M-1- or APT1-M-2-construct. Note that in cells transfected with APT1-construct APT1-fluorescence co-localized with that of Na^+/K^+ATPase (panel A, upper row). The cells transfected with APT1-M-2 construct showed increased co-localization with cell membrane marker (panel A, lower row). However, in APT1-M-1 transfected cells APT1-fluorescence predominantly localized in the cytosol and perinuclear areas (panel A, middle row). Compared to NIH3T3 cells transfected with wild type APT1 (panel B, upper row) those transfected with APT1-M-1 construct, showed increased colocalization of APT2 fluorescence with that of the membrane marker, Na^+/K^+ATPase (panel B, middle row). The cells
transfected with APT1-M-2 construct also showed increased colocalization of APT2 immunofluorescence with that of Na⁺/K⁺-ATPase (panel B, lower row).

**Figure 8.** Schematic model explaining how dynamic palmitoylation might regulate steady-state membrane localization and function of APT1 and APT2 and those of H-Ras and GAP-43. In this model, APT1 and APT2 undergo palmitoylation in the Golgi by as yet unknown PATs, which facilitates their membrane-localization. Membrane-associated APT1 and APT2 then depalmitoylate H-Ras (1) and GAP-43 (2), respectively, detaching them from the cell membrane and promoting translocation to the Golgi where they are re-palmitoylated and translocated to the membrane to manifest their function. Importantly, APT1 also depalmitoylates APT2 (3) detaching it from the membrane for another cycle of palmitoylation in the Golgi. Following depalmitoylation of APT2, APT1 then catalyzes its own depalmitoylation for translocation to the Golgi in order to undergo re-palmitoylation, required for membrane localization. However, while in this model APT1 catalyzes depalmitoylation of APT2, APT2 does not depalmitoylate APT1.
Figure 1

A

Palmitoylation site

Mouse APT1 NH2-MCGNNMSAP......COOH
Mouse APT2 NH2-MCGNNMSTP......COOH
Human APT1 NH2-MCGNNMSAP......COOH
Human APT2 NH2-MCGNTMSVP......COOH

D

E

F

G

H

I
Figure 2

A

B

| Coefficients                              | APT1     | APT1-M    | APT2     | APT2-M    |
|-------------------------------------------|----------|-----------|----------|-----------|
| Pearson's correlation coefficient (Rr)    | 0.77±0.16| 0.03±0.19 | 0.77±0.19| -0.04±0.13|
| Overlap coefficient                       | 0.99±0.04| 0.98±0.05 | 0.99±0.04| 0.91±0.03 |
Figure 3

A

| Cytosol | DMSO 0 | 25 | 50 |
|---------|--------|----|----|
| Br-Palm (µM) | + | + | + |

APT1

β-actin

1 2 3

B

| Membrane | DMSO 0 | 25 | 50 |
|----------|--------|----|----|
| Br-Palm (µM) | + | + | + |

APT1

Pan-Cadherin

1 2 3

C

DMSO

APT1

β-actin

1 2 3

Bromopalmitate

APT1

β-actin

1 2 3

D

| Cytosol | DMSO 0 | 25 | 50 |
|---------|--------|----|----|
| Br-Palm (µM) | + | + | + |

APT2

β-actin

1 2 3

E

| Membrane | DMSO 0 | 25 | 50 |
|----------|--------|----|----|
| Br-Palm (µM) | + | + | + |

APT2

Pan-Cadherin

1 2 3

F

DMSO

APT2

β-actin

1 2 3

Bromopalmitate

APT2

β-actin

1 2 3
Figure 4

A

|          | Cytosol | Membrane |
|----------|---------|----------|
| DMSO     | +       | +        |
| Palm-B (µM) | 0      | 0.5      | 1        |

Apt1

β-actin

Pan-Cadherin

1  2  3

B

|          | Cytosol | Membrane |
|----------|---------|----------|
| DMSO     | +       | +        |
| Palm-B (µM) | 0      | 0.5      | 1        |

Apt2

β-actin

Pan-Cadherin

1  2  3

C

Palm-APT1

Input

Myc

AU

D

Palm-APT2

Input

Myc

AU

E

Apt1

Na⁺/K⁺ ATPase

Merge

Magnified Inset

DMSO

Palmostatin B

F

Apt2

Na⁺/K⁺ ATPase

Merge

Magnified Inset

DMSO

Palmostatin B
Figure 7

A

APT1 | Na/K ATPase | DAPI | Merge
---|---|---|---
APT1 | | | |
APT1 M | | | |
APT1 active-site M | | | |

B

APT2 | Na/K ATPase | DAPI | Merge
---|---|---|---
APT2 | | | |
APT2 M | | | |
APT2 active-site M | | | |
Figure 8
Dynamic palmitoylation links cytosol-membrane shuttling of acyl-protein thioesterase-1 and acyl-protein thioesterase-2 with that of proto-oncogene H-Ras product and growth associated protein-43
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