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Palmitoylation of POTE family proteins for plasma membrane targeting

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Abstract

The POTE gene family is composed of 13 paralogs and likely evolved by duplications and remodeling of the human genome. One common property of POTE proteins is their localization on the inner aspect of the plasma membrane. To determine the structural elements required for membrane localization, we expressed mutants of different POTES in 293T cells as EGFP fusion proteins. We also tested their palmitoylation by a biotin-switch assay. Our data indicate that the membrane localizations of different POTES are mediated by similar 3–4 short cysteine rich repeats (CRRs) near the amino-terminuses and that palmitoylation on paired cysteine residues in each CRR motif is responsible for the localization. Multiple palmitoylation in the small CRRs can result in the strong association of whole POTES with plasma membrane.

Keywords

Prostate; Evolution; Protein modification; Plasma membrane; Palmitoylation; S-acylation; Subcellular localization; Spectrin; Ankyrin

Introduction

POTE is a primate-specific gene family composed of 13 closely related paralogs dispersed among eight chromosomes. Human genome sequence analysis suggests that all the human POTE genes evolved by duplications and remodeling of the genome from an ancestral gene, ANKRD26 [1]. POTE is expressed in many cancers (prostate, colon, lung, breast, ovary and pancreas) but in a limited number of normal organs [2–4]. The preferential expression of different POTE paralogs in certain human cancers suggests a role in carcinogenesis.

The POTE proteins vary in size but contain three distinct domains. They are amino-terminal cysteine rich domain (CRD) followed by ankyrin repeats and spectrin-like helices. CRDs are composed of 3–4 repeated CRR motifs (37-amino acid long) and form unique signatures of POTE proteins. Ankyrin repeats are present in all POTE proteins. In POTE these protein recognition modules consist of 3–7 of 33-amino acid sequence motifs [5]. The helical regions are structurally similar to the α -spectrin family of proteins which are major constituents of the

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cytoskeleton and anchored to the plasma-membrane [6]. We recently identified an actin retroposon inserted at the carboxy terminus of one of the ancestral POTE paralogs, which results in a POTE-actin fusion protein expressed in breast cancer cell lines [7].

One common property of different POTE proteins, localization on the inner aspect of plasma membrane, implies they function at the membrane. The presence of many different POTE genes on different chromosomes and their different expression profiles suggest that each paralog can function upon different physiological needs. Because the POTE ancestral gene, ANKRD26, only contains ankyrin repeat motifs and doesn't contain CRDs and the spectrin-like helices, the acquisition of the unique three domain structure in the evolution likely makes POTE family proteins express their role on membrane upon different demands.

In this study, we have addressed the molecular determinants responsible for the membrane binding of the various POTE family members. We expected that the spectrin-like helices would function for membrane localization by the analogy to spectrin family proteins. However, we found that POTE paralogs without spectrin-like helices are also localized on membranes, which prompted us to seek a different mechanism for the membrane localization of POTE proteins. Here, we demonstrate that each CRR motif (37 amino acids) can be palmitoylated to target POTEs to the membrane. Multiple palmitoylation on repeated CRR motifs could work as adaptors that connect POTEs with the membrane to function in different signaling pathways.

Materials and methods

DNA construction

EGFP cDNA from pEGFP-C1 vector (Clontech) was inserted into pCAG vector (Invitrogen) to yield pCAG/EGFP. The cDNAs encoding different POTE paralogs (GenBank accession numbers: **EF523384** for POTE2 α -action, **AY462873** for POTE2 γ c, **AY172928** for POTE21, and **AY466021** for POTE22) and their mutants were subcloned into the pCAG/EGFP vector with EGFP at the C-terminus. Cysteins were replaced with alanines site-directed mutagenesis (QuikChange, Stratagene).

Cell culture, transient transfections and microscopy

293T cells were grown in DMEM containing 10% fetal bovine serum. Transfections were performed using Lipofectamine 2000 (Invitrogen). For inhibition of palmitoylation, transiently transfected cells were treated with 2-bromopalmitate (100 μ M) overnight. Confocal imaging was performed by using a Zeiss LSM 510 laser scanning microscope.

Solubilization studies

Subcellular fractionation of the transfected 293T cells was performed by differential centrifugation [8,9]. Each fraction was analyzed by SDS-PAGE and immunoblotting using EGFP mAb (Clontech).

Radiolabeling with [3 H] palmitate

293T cells were transiently transfected with POTE-EGFP constructs. 24 h after transfection the cells growing in 60 mm dishes were starved for 1 h at 37°C in DMEM medium containing 2% dialyzed fetal bovine serum. The cells were radiolabeled in complete medium containing 50 μ Ci/ml 9,10- [3 H] palmitic acid (GE healthcare) for 16 h. The cell monolayers were then rinsed with PBS and lysed in cold RIPA buffer (50 mM Tris, pH7.5, 150 mM NaCl, 2mM EDTA 1% NP-40, 0.1 % SDS, 1% deoxycholate, 5 mM NaF and protease inhibitors). The lysates were clarified by centrifugation and the immuno-precipitated with EGFP antibody. The immuno-precipitate was washed 3 times with RIPA buffer and then 30 μ l of SDS sample buffer

was added. One μ l of the sample was added into a scintillation vial containing 5 ml of scintillation fluid and radioactivity was determined by liquid scintillation counting.

Biotin-Switch assay to detect palmitoylation

Palmitoylation of POTE was also examined by a recently developed biotin-switch assay technique [10,11]. In this protocol, acylation groups on cysteine residues via thio-ester bonds are supposed to be replaced with biotins. 293T cells were transiently transfected with POTE-EGFP constructs and scraped 48 h after the transfection. The cells were pelleted and disrupted in 600 μ l of lysis buffer (PBS/1% Triton X-100/25 mM *N*-ethylmaleimide/5 mM EDTA) by sonication. After blocking of the free cysteines by 30-min incubation on ice, proteins were precipitated with methanol/chloroform. The air-dried pellet was resuspended in 100 μ l of resuspension buffer (2% SDS/8 M urea/100 mM NaCl/50 mM Tris-HCl, pH 7.4) by sonication, diluted with 600 μ l of 1 M hydroxylamine (pH 7.4) and 300 μ M biotin-BMCC (Pierce), and rotated for 2 h at 4°C. As a control, hydroxylamine was replaced with 50 mM Tris pH 7.4 to avoid the removal of the *S*-acylated (palmitoylate) groups for further biotinylation. Biotinylated proteins (previously palmitoylated proteins) in samples were precipitated with 100 μ l of neutravidin-agarose beads (Pierce) in 1 ml of PBS containing 0.1% TritonX-100. After washing, proteins were eluted by boiling for 5 min in 20 μ l of resuspension buffer plus 40 ml of 4x SDS sample buffer lacking 2-mercaptoethanol. Samples were subjected to SDS/PAGE and analyzed by western blotting using a monoclonal EGFP antibody (Clontech).

Results

Membrane localization of different POTE paralogs expressed in 293T cells

To confirm the plasma membrane localization of each POTE paralog as EGFP fusion proteins, 3 cDNAs encoding POTE21, POTE2 γ C and POTE22 were expressed in 293T cells. These 3 POTE proteins were chosen based on their relative abundance in tissues and cancers [2] and on the previous substantial characterization of the recombinant protein without EGFP [4]. All the POTE-EGFP proteins showed signals concentrated on the plasma membrane, whereas the signal from EGFP-only control was observed in cytoplasm and nucleus (Fig. 1A). A similar localization of each protein was also observed in transfected KB cells (data not shown).

Cysteine rich domain is required for membrane localization of POTE paralogs

POTE proteins usually contain three major domains: CRDs, ankyrin repeat motifs, and spectrin-like helices. To examine the contribution of each to the membrane localization, we separately expressed these domains from different POTE paralogs as EGFP fusions. Fig. 1B shows the result with POTE21-derived truncated mutants. CRD from POTE21 were localized at plasma membrane, whereas the ankyrin repeats were localized in cytoplasm and nuclei and the spectrin-like helices were expressed in the cytoplasm. A mutant containing both ankyrin repeats and the spectrin-like helices was also expressed in cytoplasm. Similar results indicating the need of the CRDs for the plasma membrane localization were obtained with the other three paralogs (Fig. 1C). We conclude that the CRDs are sufficient for the association of POTE with the plasma membrane.

Each cystein rich domain contains the structural elements for membrane localization

The CRD domain of each POTE paralog is made up of 3–4 repeats of ~37 aa each [cysteine rich repeat (CRR)]. The sequences of these repeats are similar but not identical. Twenty different CRR sequences from POTE-2 α , 2 γ , 8, 14, 15, 21, and 22 genes show 54–97 % of identity. To characterize the membrane targeting motifs, we expressed each CRR motif from different POTE paralogs as EGFP fusions and examined their localization. Fig. 2A shows the localization of each CRR motif from POTE21-EGFP protein (CRR-1, CRR-2 and CRR-3).

With the exception of CRR-1 of POTE22 (Fig. 2A, last panel), all the CRR motifs resulted in membrane localization of their EGFP-fusion proteins (Fig. 2B).

Possible palmitoylation of cysteine residue(s) in each CRR motif

To assess the nature of the membrane association of the recombinant POTE CRRs, we performed solubilization studies with CRR-1/POTE21 assuming that the membrane binding mechanisms of all CRRs of POTE are similar. We found that CRR-1/POTE 21 was present mostly in the membrane fraction, consistent with our finding that CRR1/POTE 21 is a membrane protein (Fig. 2A). The POTE protein was solubilized only in 1% Triton X-100 and not in 0.5 M NaCl, 0.5M EDTA or 2 M urea (data not shown). This suggests that the tight association of POTE with membranes could be due to a possible post translational modification such as lipidation and is not due to a hydrophilic interaction or electrostatic or ionic interaction via a calcium ion with lipid or with other membrane proteins [8,9].

Each CRR motif contains a cysteine cluster (3–6 cysteines in a 10 amino acid string) in the middle of a sequence accompanied by 3–5 positively charge amino acids, consistent with palmitoylation of these cysteine residues [12–14]. Although there is no common canonical consensus sequence for palmitoylation [12,15], a computer algorithm predicts that the cysteines that can be palmitoylated based on clustering and scoring of known palmitoylated sites on proteins (CSS-Palm, http://bioinformatics.lcd-ustc.org/css_palm/ [16]. A comparison of sequences of various CRR motifs and the results of the prediction using CSS-Palm is shown in Fig. S1. The program predicted 1–4 cysteines for palmitoylation in 11 out of 13 CRR motifs using a threshold for prediction corresponding to 83% specificity and 82% sensitivity [16]. The absence of a predicted palmitoylated cysteine in POTE22-CRR-1 agrees with our observation that this CRR motif does not localize to the membrane (Fig. 2A, last panel).

To determine if CRR-1 of POTE21 is modified by acylation with palmitate, we measured its endogenous acylation state using the biotin-switch method [10,11]. In this method, hydroxylamine cleaves the thio-ester bond generating a free cysteine, which can be modified with maleimide conjugated with biotin (biotin –BMCC). The biotin modified protein is precipitated with neutravidin and detected using western blots. As shown in Fig. 3B, biotinylated POTE2 α -EGFP, POTE2 γ c-EGFP and POTE21-EGFP were detected by an anti-EGFP antibody around the expected sizes depending on the addition of hydroxylamine. Similarly, the acylation via thio-ester bonds was also detected in POTE21-CRR-1-EGFP (Fig. 3A, last two lanes). Faint bands corresponding to inefficiently biotinylated proteins were detected for POTE2 γ c-EGFP and POTE21-CRR-1-EGFP (Fig. 3A) probably because of the relatively higher expression level of these proteins compared to POTE 21 and POTE2 α (Fig. 3B). These results indicate that POTE proteins are expressed as acylated protein in the 293T cells.

To verify this conclusion we performed metabolic labeling with [3 H]-palmitate of the 293T cells transfected with a POTE21-CRR-1-EGFP plasmid. The labeled protein was immunoprecipitated using EGFP antibody and its radioactive content measured. Cells transfected with an EGFP plasmid served as controls. As shown in Fig. 3C, the immunoprecipitate from POTE21-CRR-1-EGFP showed >10 fold higher radioactivity than the control confirming that the CRRs are expressed as palmitoylated proteins.

An inhibitor of palmytoylation abolished the membrane localization of POTES

To determine if the CRRs of POTE are localized to the membrane by palmitoylation, we blocked palmitoylation by addition of 2-bromopalmitate, an inhibitor of palmitoylation [17]. Fig. 3D shows images of POTE2 γ c-EGFP, POTE21-EGFP, and POTE21-CRR-1-EGFP with or without 100 μ M 2-bromopalmitate. The palmitoylation inhibitor treatment abolished

plasma-membrane localization of all 3 proteins and they moved into the cytosol. The difference in subcellular distribution was not due to a difference in expression level, because comparable amounts of each POTE protein were expressed in 293T cells based on western blot analysis using EGFP antibody (Fig. 3E). This suggests that POTE is targeted to the plasma-membrane through the palmitoylation of the CRR motifs.

Mutagenesis in tandem cysteine residues in each CRR motif abolished the membrane localization

Mutagenesis of cysteine(s) to alanine(s) is a widely used to identify cysteine residues for palmitoylation [18]. Because different CRR motifs contain multiple paired and unpaired cysteines (Fig. 3A), the palmitoylation must occur at cysteines at different positions of different CRR motifs. Inspection of the CRR sequences revealed that all the CRR motifs that associate with the plasma membrane contain at least one paired cysteines. Most of the paired cysteines were predicted to be palmitoylated by CSS-Palm software. The only CRR that does not contain paired cysteines (POTE22-CRR-1) did not localize to the membrane (Fig. 2A, last panel). In addition, cysteine residue is most commonly observed next to the palmitoylated cysteine in many proteins [19]. To examine the palmitoylation of CRR motifs, we mutated various paired and unpaired cysteines.

Fig. 4A shows the localization of a series of mutants in which 3 cysteine residues of CRR-1 of POTE21 were changed to alanine (an unpaired cysteine at position 28 and paired two cysteins at position 34/35). C28A-CRR-1-EGFP was localized at the plasma membrane as well as non-mutated CRR-1-EGFP but C34A/C35A-CRR-1-EGFP lost its membrane localization and moved to the cytoplasm. Thus this CRR was palmitoylated on the paired cysteine residues. When C34A/C35A-CRR-1 was introduced in a whole POTE21 protein with unmutated CRR-2 and CRR-3, the protein was localized to the membrane (data not shown), indicating each CRR motif can act independently as a membrane-binding module.

Different CRR motifs such as CRR-2/POTE21 possess 5 cysteine residues, 4 of them making two pairs (Fig. 3A). To examine palmitoylation on cysteines of this type of CRR, we generated two mutants with one paired cysteine mutated to alanine (C64A/C65A-CRR2 and C71A/C72A-CRR-2) and one mutant with both paired cysteines mutated (C64A/C65A/C71A/C72A-CRR-2). As shown in Fig. 4B, mutants C64A/C65A-CRR-2 and C71A/C72A-CRR-2 still exhibited plasma-membrane localization whereas the mutant in both paired cysteines, C64A/C65A/C71A/C72A-CRR-2, didn't bind to the membrane. This result suggests that this CRR motif undergo palmitoylation at the 2 paired cysteine residues and the palmitoylation on either of the paired cysteines is sufficient for targeting the CRR motif to the membrane.

Discussion

This study analyzes amino acids necessary for the membrane binding of several POTE paralogs. We found that the CRD is responsible for targeting the POTE paralogs to the membrane. Because the CRDs can be divided into 3 or 4 repeats (CRR) of 37 amino acids each, we generated proteins where each of the repeats was fused with EGFP. We found that each CRR motif of POTE paralogs was localized in the plasma membrane, except the CRR-1 motif of POTE22. This finding suggests that each of the CRR motifs can target the POTE paralogs to the membrane and can behave as an independent membrane-targeting module. Further, using biochemical and mutational analysis, we found that CRR motifs are palmitoylated at the paired cysteine residues. This suggests that many POTE CRR motifs can be localized at the plasma membrane despite of the lack of common canonical sequence.

In general, the primary role of protein palmitoylation is facilitating membrane interactions [20]. Recent studies have shown that palmitoylation acts as a trafficking signal for H-Ras and

N-Ras and is responsible for targeting the protein to the plasma-membrane through the exocytic pathway [21]. In addition, accumulated evidences suggests multiple roles of palmitoylation in modulating protein-protein interactions and enzyme activity [15]. These palmitoylated proteins include functionally diverse proteins such as neuronal proteins, [22–24], cancer promoting and suppressing proteins [25], and G-protein linked receptors [26,27]. In these proteins, palmitoylation is usually accompanied by other lipid anchors or transmembrane domains that associate with membranes. In POTE proteins, there are no such domains for additional interactions with membranes, but multiple CRR motifs in each POTE can form multiple palmitoylation groups to strengthen binding to the membrane.

Repeated CRR units represent a new type of palmitoylation motif. Database searches using CRR sequences did not hit similar sequences from other genes, suggesting that CRR motifs are likely associated with localization mechanisms specific for POTE proteins. It is likely that the multiple palmitoylation of the CRR motifs mediates a tight association of the protein with the plasma membrane and the ankyrin and α -helical repeats are responsible for protein-protein interaction(s). Once bound to the membrane, POTE acts as a scaffold, where signaling proteins interact and are responsible for downstream targeting. We are currently trying to identify the binding partners of POTE to learn more about the function of POTE paralogs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Intracellular localization of different POTE paralogs and domains in 293T cells as EGFP-fusions. (A) POTE21-EGFP, POTE2 γ C-EGFP, POTE22-EGFP and control EGFP were expressed in 293T cells and live cells were monitored by confocal microscopy. EGFP fluorescence (green) and nuclei counterstained with DAPI (blue) were shown. PM, plasma membrane; CY, cytoplasm, N, nucleus. (B) Localization of each domain of POTE21 expressed in 293T cells. The amino acids numbers at the borders of each construct are shown on the POTE21 structure model. (C) A summary of intracellular localization of each domain from different POTE paralogs. NT, not tested; -, not existing. All figures are shown with the same magnification with the scale bar shown in the first panel of Fig. 1A.

Fig. 2.

Subcellular localization of each CRR motif from POTE paralogs. (A) Localization of CRR1, CRR2 and CRR3 from POTE21 and CRR1 from POTE22 as EGFP fusion proteins. (B) A summary of localization of each CRR from different POTE paralogs. PM, plasma membrane; CY, cytoplasm, N, nucleus.

Fig. 3.

Palmitoylation of CRR motifs responsible for plasma membrane targeting. (A) Palmitoylation (S-acylation) of POTE-EGFP proteins detected by a biotin switch assay. After the transfection, total cell lysates were prepared and treated with N-ethylmaleimide to block free cysteines. The protein were treated (+) or untreated (-) with hydroxylamine to remove the palmitate. The free cysteine residues were biotinylated with biotin-BMCC. The biotinylated proteins (palmitoylated proteins) were precipitated with neutravidin beads for the immunoblotting analysis. A MA b to EGFP was used for the detection of EGFP-fusion proteins in the western analysis. Equal amounts of total protein were loaded in each lane. (B) Expression level of POTE proteins expressed in 293T cells examined by immunoblotting with EGFP antibody. The samples are: lane 1: POTE21/CRR1-EGFP, lane 2: POTE2γC-EGFP, lane 3: POTE21-EGFP, lane 4: POTE2α-EGFP. The blot was probed with anti-actin as a loading control. (C) Graph showing the incorporation of [³H] 9,10 palmitic acid into the recombinant proteins. Metabolic labeling of the 293T cells with [³H] 9,10 palmitic acid was performed as described in the Materials and Methods. EGFP fusion protein was immunoprecipitated using anti-EGFP polyclonal antibody and the radioactivity was measured in a liquid scintillation counter. Samples were set up in triplicate. EGFP expressed in the 293T cells served as a negative control. (D) Effect of an inhibitor of palmitoylation on sub-cellular localization of POTE paralogs. The 293T cells were transfected with POTE-EGFP fusion proteins and incubated with or without 100 μM of 2-bromopalmitate. (E) Expression level of POTE proteins remains unchanged after the treatment of 2-bromopalmitate as examined by immunoblotting with EGFP antibody.

Fig. 4.

Effects of mutation of cysteine to alanines in CRR motifs on subcellular localization. (A) Mutants of POTE21-CRR1-EGFP fusion proteins. The paired cysteines (C34/C35) were responsible for the plasma membrane localization. (B) Mutants of POTE21-CRR2-EGFP fusion proteins. Both of the two paired cysteines (C62/C62 and C71/C72) are responsible for the membrane localization.