Development of a Novel Quantitative Method for Protein *S*-Palmitoylation and Exploration of Depalmitoylating Enzymes for PSD-95

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Introduction

Reversibly regulated post-translational modifications of proteins such as protein phosphorylation play central roles in various cellular events. *S*-palmitoylation, also called *S*-acylation, is another representative reversible modification of proteins. *S*-palmitoylation is an addition of a fatty acid, mainly 16-carbon saturated palmitate, to a sulfhydryl group of a specific cysteine residue via a thioester linkage. Recent global proteome studies have greatly expanded knowledge about the numbers and types of palmitoyl proteins, such as signaling molecules, enzymes, cytoskeletal proteins, ion channels and cell adhesion molecules, and so on. Intriguingly, palmitoylated proteins significantly accumulate in specialized membrane structures of the cell, called membrane microdomains such as immunosynapses of immune cells, tight junctions of epithelial cells, and pre/post-synapses in neurons. Increasing evidence proposes that *S*-palmitoylation is one of the most important factors of formation and maintenance of membrane microdomains.

Reversible *S*-palmitoylation is catalyzed by two types of enzymes, palmitoyl acyltransferases (PATs; *i.e.*, palmitoylating enzymes) and palmitoyl-protein thioesterases (PPTs; *i.e.*, depalmitoylating enzymes). Asp-His-His-Cys (DHHC) motif-containing DHHC family members function as evolutionally conserved PATs. On the other hand, PPTs remain controversial. Thus, the regulatory mechanism of reversible *S*-palmitoylation cycles is incompletely understood. Another important issue in the *S*-palmitoylation research is the lack of the quantitative method to address *S*-palmitoylation stoichiometries of proteins in cells or tissues.

Materials and Methods

All animal studies described herein were reviewed and approved by the ethics committee at National Institute for Physiological Sciences and were performed according to institutional guidelines concerning the care and handling of experimental animals.

Cloning of serine hydrolase genes and plasmid construction

Total RNAs were extracted by Trizol reagent (Life technologies) from mouse and rat whole brains and purified by isopropanol precipitation. Complementary DNAs (cDNAs) were obtained by reverse transcription-polymerase chain reactions (RT-PCRs) with Superscript-III system (Life technologies). cDNAs of serine hydrolase genes were amplified by specific primer sets and subcloned into pCAGGS-Flag(N), pCAGGS-HA(N), and pEGFP-N. All PCR products were analyzed by DNA sequencing.

Cell culture and transfection

HEK293T cells

HEK293T cells were grown in Dulbecco's Modified Eagle Medium (DMEM, Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich) at 37^{o}C with 5% CO_2. After 14-20 hours, HEK293T cells were transfected with plasmid DNAs by Lipofectamine and plus reagent system (Life technologies).

Cultured rat hippocampal neurons

Rat hippocampal neurons were isolated from E18-19 embryonic rats (Wistar/ST). Neurons were cultured in neurobasal medium supplemented with B-27 supplement (Life technologies) and 2 mM Glutamax (Life technologies) at 37°C with 5% CO_2. Cultured neurons were transfected with plasmid DNAs by Lipofectamine 2000 (Life technologies).

Metabolic [^{3}H]-palmitate labeling assay

HEK293T cells (2.5 x 10^5 cells) were seeded in 12 well-plates. Cells were co-transfected PSD-95-GFP with individual candidate clones. After 24 hours, cells were preincubated for 30 min in serum-free DMEM with fatty acid-free bovine serum albumin. Then, cells were labeled with [^{3}H]-palmitate for 4 hours in the preincubation medium. Cells were washed with PBS at room temperature, lysed by 0.5 ml of SDS-PAGE sample buffer, and boiled at 90°C for 2 min. For fluorography, proteins were separated by SDS-PAGE. Gels were fixed by the fixation solution (25% isopropanol and 10% acetic acid) for 20 min and treated with the fluorography solution (1 M sodium salicylate and 15% ethanol) for 20 min to amplify the radioisotope signal. After gel drying, gels were exposed to X-ray films at -80°C.

Fluorescence microscopy

For GFP-fusion proteins, transfected cultured neurons (2.5 x 10^4 cells in 24-well plate; 21-24 DIV) were fixed with 4% paraformaldehyde/120 mM sucrose/100 mM 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid (HEPES) pH 7.4 at room temperature for 10 min. Fluorescent images were acquired with a confocal laser scanning microscopy system (TCS SP5 II; Leica) equipped with an HCXPL APO 63x/1.40 oil immersion objective lens, combined with the Leica HyD detector.

Immunoprecipitation

Neurons expressing HA-tagged PSD-95 PPT (P-PPT) candidate protein were treated by lysis buffer for 20 min on ice. Collected cell lysates were clarified by the centrifugation at 11,000 x g for 10 min and supernatants were incubated with anti-HA antibody (12CA5) for 1 hour. Then, protein A-Sepharose (GE healthcare) was added and this mixture was incubated for 1 hour. Precipitated beads were treated with 150 μl of Laemmli SDS-sample buffer with 2% 2-mercaptoethanol and boiled at 100 ^{o}C for 5 min.

Real-time quantitative PCR

Total RNA of cultured hippocampal neurons was extracted by the Trizol method. cDNA was synthesized using the High-Capacity cDNA RT kit (Life technologies). Real-time PCR was performed using an Applied Biosystems StepOnePlus^{TM} real-time PCR system (Life technologies) and SYBR Green PCR Fast master mix (Life technologies). I used two independent primer pairs for individual target genes.

Results

Chapter 1

Development of a novel quantitative method for *S*-palmitoylation

Although recent palmitoyl-proteome analysis identified a substantial number of palmitoyl proteins, no methods have addressed the number of palmitoylated cysteine residues in proteins and their palmitoylation stoichiometries *in vivo*. Here, I developed a novel quantitative method which comprises sequential four chemical reactions: 1) reduction of intra- and intermolecular disulfide bonds by tris-(2-carboxyethyl) phosphine (TCEP); 2) blockade of free cysteine thiols with *N*-ethyl maleimide (NEM); 3) hydroxylamine treatment to cleave palmitoyl-cysteine thioester linkages; and 4) introduction of maleimide-conjugated polyethylene glycols (mPEGs) into newly exposed cysteine thiols, which are originally *S*-palmitoylated cysteines. I first selected the optimal mPEGs and checked each reaction condition to precisely observe palmitoylation stoichiometries. Then, I attempted to verify the number and stoichiometry of *S*-palmitoylation of various endogenous proteins including PSD-95 in cultured rat hippocampal neurons. I found that about 80% of PSD-95 was di-palmitoylated and that mono-palmitoylated form of PSD-95 was hardly detected in neurons. So far, the half-life of palmitate on PSD-95 was shown to be approximately 2 hours by an [^{125}I]-palmitate pulse-chase experiment, although the detailed processes, dynamic changes between two palmitoylated states, remain unclear. Taking advantage of the APEGS assay, I found that the half-life of di-palmitoylated PSD-95 was calculated to be 1.84 ± 0.46 hours. Furthermore, the mathematical simulation suggests that the palmitoylation reaction of PSD-95 is four times as fast as the depalmitoylation reaction.

Chapter 2

Exploration of novel depalmitoylating enzymes for PSD-95

Although *S*-palmitoylation is one of the most common "reversible post-translational modifications", the enzymes that mediate depalmitoylation have been controversial over 35 years. Because thioesterases mainly belong to huge serine hydrolase superfamily including proteases, esterases, and lipases, I attempted to examine whether members of uncharacterized serine hydrolases have depalmitoylation activities. Here, to identify PSD-95 palmitoyl-protein thioesterases (P-PPTs; *i.e.*, PSD-95 depalmitoylating enzymes), I isolated cDNAs of 38 serine hydrolase genes. I first examined effects of candidate proteins on the palmitoylation level of PSD-95 by a metabolic labeling assay with [^{3}H]-palmitate in HEK293T cells. The co-expression of several clones dramatically reduced palmitoylation levels of PSD-95-GFP. Then, I obtained 10 genes as initial candidates for P-PPTs. To further examine the functional similarity of these candidate proteins, I generated the phylogenetic tree of P-PPT candidates and found that candidate P-PPTs can be classified into several subfamilies. I confirmed these candidate genes were expressed in the cultured rat hippocampal neurons by real-time quantitative PCR. I next investigated subcellular localizations of GFP-fused P-PPT proteins in rat hippocampal neurons. Three members were uniquely localized in dendritic vesicles and dendritic spines. Because PSD-95 is depalmitoylated mainly at post-synaptic membranes, I focused on post-synaptic P-PPT candidates. To examine whether post-synaptic P-PPTs have hydrolase activities, I explored catalytic amino acids of post-synaptic P-PPTs using the *in silico* predicted structure and experimentally identified Ser, Asp, and His as catalytic residues for their hydrolase activity. Next, I investigated the function of post-synaptic P-PPTs in neurons. When a member of post-synaptic P-PPT was overexpressed in rat

hippocampal neurons, post-synaptic P-PPT directly bound to PSD-95 and dramatically reduced the palmitoylation level of endogenous PSD-95 in neurons. Finally, I investigated whether post-synaptic P-PPTs mediate depalmitoylation of endogenous PSD-95 in neurons. For this purpose, post-synaptic P-PPTs were knocked down by triple infections of individual miRNA-encoding AAVs in rat hippocampal neurons. However, the depalmitoylation process of PSD-95 was not changed by the knockdown of post-synaptic P-PPTs under the present experimental condition.

Discussion

The APEGS assay I developed in this study is very powerful for the *S*-palmitoylation analysis as this method for the first time provides information about the number of *S*-palmitoylation sites and the stoichiometry of palmitoyl proteins. Given that cysteine residues are subjected to various post-translational modifications such as nitrosylation, sulfhydration, and so on, I expect that the principle of the APEGS assay could be widely applied to these cysteine modifications.

Three post-synaptic P-PPTs are the most promising candidates for PSD-95 depalmitoylating enzymes as post-synaptic P-PPTs interacted with PSD-95 and robustly reduced the palmitoylation level of PSD-95 in neurons. However, the triple knockdown of post-synaptic P-PPTs in neurons did not show any effects on the depalmitoylation process of PSD-95, probably due to the incomplete knockdown, the molecular redundancy of P-PPTs or the existence of other authentic enzymes. In my screening, at least ten proteins showed depalmitoylating activities for PSD-95. Therefore, it is worthwhile to examine the effects of multiple knockdown or knockout of these different P-PPT subfamilies.

In conclusion, I first developed a novel quantitative method for protein *S*-palmitoylation to quantitatively understand the property of the palmitate cycling on PSD-95. I for the first time succeeded in quantifying the palmitoylation stoichiometry of PSD-95 in neurons and the dynamic depalmitoylation process of PSD-95. In addition, I explored PSD-95 PPTs and obtained promising candidates.