Local palmitoylation cycles define activity-regulated postsynaptic subdomains

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Introduction

Organization of specialized membrane domains, such as focal adhesions, tight junctions, and pre/post-synapses, requires ordered protein assembly at the plasma membrane. In neurons, proteins that mediate synaptic vesicle fusion and neurotransmitter release concentrate specifically at the presynaptic active zone. Correspondingly, neurotransmitter receptors, postsynaptic scaffolding proteins, and various signaling proteins precisely align at a specialized postsynaptic membrane region, called the postsynaptic density (PSD). The size, shape, and protein composition of the PSD determine the function of individual excitatory synapses.

PSD-95 is the most abundant scaffold protein specifically enriched in the PSD. It contains three PDZ domains, an SH3 domain, and a guanylate kinase (GuK)–like domain (Kim and Sheng, 2004; Funke et al., 2005). Through its PDZ domains, PSD-95 assembles at the PSD various synaptic components including intracellular signaling molecules (e.g., SynGAP and kalirin-7), ion channels (e.g., stargazin/AMPA receptors [AMPARs] and NMDA receptors), and cell adhesion molecules (e.g., neuroligin). The following observations indicate that PSD-95 plays a primary role in synaptic development and maturation: (1) PSD-95 clusters at synapses before other postsynaptic proteins (Rao et al., 1998) and lies closer to the postsynaptic membranes than other proteins (Valthschannoff and Weinberg, 2001; Dani et al., 2010); (2) reduced expression of PSD-95 causes patchy loss of the PSD area (Chen et al., 2011); and (3) overexpression of PSD-95 can drive maturation of glutamatergic synapses (El-Husseini et al., 2000b). In addition, PSD-95 regulates synaptic transmission and plasticity by regulating the molecular composition of the PSD, including palmitoylation activity, ensuring the maintenance of compartmentalized PSD-95 clusters within individual spines. Plasma membrane targeting of DHHC2 palmitoyltransferase rapidly recruited PSD-95 to the plasma membrane and proved essential for postsynaptic nanodomain formation. Furthermore, changes in synaptic activity rapidly reorganized PSD-95 nano-architecture through plasma membrane–inserted DHHC2. Thus, the first genetically encoded antibody sensitive to palmitoylation reveals an instructive role of local palmitoylation machinery in creating activity-responsive PSD-95 nanodomains, contributing to the PSD (re)organization.
the number of synaptic AMPARs (El-Husseini et al., 2000b; Béïque et al., 2006; Ehrlich et al., 2007; Elias and Nicoll, 2007). Protein–protein interactions and palmitoyl lipid modification each play important roles in the postsynaptic targeting of PSD-95 (Craven et al., 1999). The neurexin–neuroligin trans-synaptic interaction triggers PSD-95 recruitment through a PDZ-domain–mediated interaction (Graf et al., 2004; Chih et al., 2005; Nam and Chen, 2005). However, neurons derived from neuroligin triple knockout mice still show normal synaptic accumulation of PSD-95 (Varoqueaux et al., 2006). Palmitoylation of PSD-95 at its N-terminal cysteine residues is essential for its postsynaptic targeting, as a palmitoylation-deficient PSD-95 mutant is diffusely distributed in dendrites and the cell body (Topinka and Bredt, 1998). It remains still unclear, however, what defines the location of PSD-95 clusters, and how PSD-95 clusters are maintained and remodeled to regulate the organization of the entire PSD.

Protein palmitoylation is a frequent lipid modification that regulates protein trafficking to intracellular or plasma membranes (El-Husseini Ael and Bredt, 2002; Resh, 2006; Linder and Deschenes, 2007; Fukata and Fukata, 2010; Salaun et al., 2010; Sen and Snyder, 2010). This modification is reversibly catalyzed by DHHC-type palmitoyl acyltransferases (PATs) and still uncharacterized depalmitoylating enzymes. Recent live-cell imaging experiments using fluorescently tagged palmitoyl substrates (e.g., H-Ras, Go, and PSD-95) and a palmitoylation inhibitor revealed a role for palmitoylation in the dynamic relocalization of palmitoyl proteins between membrane compartments (Rocks et al., 2005; Chisari et al., 2007; Noritake et al., 2009; Tsutsumi et al., 2009). However, exogenously expressed fluorescent proteins may not necessarily behave like their endogenous counterparts and the use of overexpressed proteins may cause more subtle physiological properties to be overlooked.

Here, we developed a novel probe for spatiotemporally visualizing the palmitoylation state of endogenous PSD-95. This probe revealed an important role of local palmitoylation cycles to functionally organize activity-responsive nanodomains of PSD-95 in the postsynapse.

Results

Selection of a palmitoylation-specific recombinant antibody against PSD-95, PF11

To visualize the palmitoylation state of endogenous PSD-95 in fixed and in living cells, we generated a palmitoylation-specific biosensor. We took advantage of the antibody phage display system, which has allowed selection of powerful conformation-specific recombinant antibodies (Nizak et al., 2003b; Dimitrov et al., 2008). We screened a phage display library of recombinant single-chain variable fragment (scFv) antibodies against palmitoylated PSD-95. The antigen was purified from HEK293T cells coexpressing PSD-95-GFP fused to PSTCD, a biotin acceptor peptide, and one of PSD-95 PATs, DHHC15 (with DHHC2, 3, and 7; Fig. 1, A–C; Fukata et al., 2004). The subsequent antibody selection screen yielded a promising scFv clone, named PF11 (Fig. 1 C). In fixed hippocampal neurons, hPF11 (PF11 fused to human IgG Fc) specifically stained endogenous PSD-95 clusters, as indicated by the lack of staining in PSD-95 knockdown neurons (Fig. 1 D). In adult mouse brain sections, hPF11 antibody showed strong punctate signals in the neuropil of, for example, hippocampus and cerebellum, and its overall staining pattern was consistent with that of a conventional PSD-95 antibody (Fig. 1 E).

We next tested whether PF11 distinguishes palmitoylated PSD-95 from nonpalmitoylated PSD-95. When expressed with DHHC2 in HEK293T cells, wild-type PSD-95 (PSD-95 WT-GFP) was found at the plasma membrane (Fig. 1 F), where it was recognized by hPF11. In contrast, a palmitoylation-deficient PSD-95 mutant, in which the N-terminal palmitoyl cysteines 3 and 5 were mutated to serines (PSD-95 CS-GFP), was diffusely distributed in the cytoplasm and not stained with hPF11 (Fig. 1 F). PF11 specificity for palmitoylated PSD-95 was confirmed in hippocampal neurons using a molecular replacement strategy. Expression of endogenous PSD-95 was reduced via shRNA knockdown and replaced by expression of either a PSD-95 WT or PSD-95 CS; both constructs were shRNA resistant (Fig. 1 G). PSD-95 WT was localized as clusters at dendritic spines and clearly labeled by hPF11. In contrast, PSD-95 CS was diffusely distributed in the cell body and dendrites and was not recognized by hPF11. Thus, PF11 recognition of PSD-95 expressed in cells depends on PSD-95 palmitoylation.

We compared signals of hPF11 (representing palmitoylated PSD-95) with those of a conventional PSD-95 antibody (representing total PSD-95) in neurons. hPF11 signals were specifically detected as small discrete puncta in dendrites and hardly detected in the soma (Fig. 2, A and B). Nearly all dendritic hPF11 signals overlapped with or were closely apposed to a presynaptic marker protein, vGlut1 (Fig. 2 B, shown by asterisks). In contrast, total PSD-95 signals were occasionally present outside synaptic sites in both dendrites and the soma (Fig. 2, A and B, with neither vGlut1 nor hPF11 signals shown by arrows). When virtually all the hippocampal glutamatergic excitatory synapses were labeled with the mixture of two antibodies against vGlut1 and vGlut2 (vGlut), 97.4 ± 1.2% of hPF11 and 89.9 ± 1.5% of total PSD-95 signals were colocalized or closely apposed with vGlut signals in dendritic regions (P < 0.001, Student’s t test; five neurons, ~2,000 clusters), indicating that hPF11 antibody exclusively labels postsynaptic PSD-95 clusters. We observed a higher intensity ratio of dendritic spines vs. soma fluorescence when staining neurons with hPF11 (ratio, 19.5 ± 4.5) than when staining total PSD-95 (ratio, 6.7 ± 1.9; P < 0.001, Student’s t test; 6 neurons). Upon treatment with 2-bromopalmitate (2-BP), which inhibits protein palmitoylation, hPF11 signals at the spines were reduced sensitively, but signals in the soma were not reduced (Fig. 2, C and D). These results directly demonstrate for the first time that palmitoylated PSD-95 is almost exclusively localized at excitatory synapses in neurons and that depalmitoylation of PSD-95 specifically occurs at the synapse.

PF11 as an intrabody tracks palmitoylated PSD-95 conformation in living cells

Next, we examined PF11’s ability to recognize palmitoylated PSD-95 in living cells. PF11 cDNA was fused with GFP cDNA and used as a fluorescent intracellular antibody (Fig. 1 C), as described previously (Nizak et al., 2003b). This intrabody was coexpressed with PSD-95-mCherry in HEK293T cells (Fig. 3, A–F).
Figure 1. Selection of PF11, a recombinant antibody specific for palmitoylated PSD-95. (A) PSD-95-GFP-PSTCD metabolically labeled with \[^{3}H\}\)palmitic acid in HEK293T cells was analyzed by fluorography \(^{3}H\)palm and Coomassie brilliant blue (CBB) staining. Closed and open arrowheads indicate the positions of palmitoylated and nonpalmitoylated PSD-95, respectively. (B) Palmitoylated PSD-95-GFP-PSTCD was purified to near homogeneity. (C) A combinatorial recombinant antibody library was screened in vitro against palmitoylated PSD-95. A promising clone, PF11, was obtained. (D) Indirect PF11 immunofluorescence. The signal (hPF11, red) disappeared in PSD-95 knockdown neurons (shPSD-95, green, arrows). Bars, 20 µm (5 µm, magnified). (E) hPF11 (green) and PSD-95 (red) antibodies showed the similar staining pattern in the hippocampus (top) and cerebellum (bottom) of adult mouse brain sections. Glutamatergic presynapses were labeled with the mixture of vGlut1 and vGlut2 antibodies (blue). DG, dentate gyrus; Mo, molecular layer; PC, Purkinje cell layer; Gr, granule cell layer. Regions in the molecular layer of DG and cerebellum are magnified (right panels). Bars: (top) 500 µm; (bottom left) 100 µm; (bottom right) 2.5 µm. (F) HEK293T cells were cotransfected with DHHC2 and either PSD-95 wild-type (WT) or cysteine-mutated palmitoylation-deficient PSD-95 CS, and cells were stained with hPF11 (red). Bars, 10 µm. (G) hPF11 staining (red) was detected in neurons expressing shPSD-95 (marked by GFP, blue pseudocolor) and complemented by shRNA-resistant (res) PSD-95 WT (stained by anti-PSD-95, green pseudocolor), but not by palmitoylation-deficient resPSD-95 CS. Bars, 10 µm (1 µm, dendritic spines magnified).

Under control conditions, PF11-GFP was diffusely dispersed throughout the cell (Fig. 3 A). In the presence of DHHC2, PF11-GFP and PSD-95-mCherry cotranslocated to the plasma membrane or occasionally to vesicular transport intermediates (Fig. 3, B and C). An intrabody against a nonrelated protein, CC7-GFP, remained dispersed throughout the cell when expressed...
but was not recognized by PF11-mCherry (Fig. S1 B), which indicates that PF11 binding requires N-terminal modification of PSD-95. As expected, GAP43, which is another N-terminally palmitoylated protein, was not recognized by PF11 (Fig. S1 C) and PF11 did not react with palmitoylated PSD-93, which is closely related to PSD-95 (Fig. 3 F). Importantly, PF11 recognized PSD-95 chimeric constructs in which the short N-terminal palmitoylation motif of PSD-95 had been replaced with that of GAP43 (+GAP43-N11, Fig. S1 D) or with another type of acylation motif (the N-terminal myristoylation motif of Src, +Src-N20; Fig. S1 E). Furthermore, the antibody did not react with either a short palmitoylated motif from PSD-95 (N13, 1–13 aa) or with palmitoylated 1-SH3 (1–495 aa), which lacked the GuK domain (Fig. 3 F). In contrast, PSD-95 d(PDZ1,2), which lacked the first and second PDZ domains, was detected by PF11 (Fig. S1 F) but not by PF11-mCherry (Fig. S1 B), which indicates that PF11 binding requires N-terminal modification of PSD-95. As expected, GAP43, which is another N-terminally palmitoylated protein, was not recognized by PF11 (Fig. S1 C) and PF11 did not react with palmitoylated PSD-93, which is closely related to PSD-95 (Fig. 3 F). Importantly, PF11 recognized PSD-95 chimeric constructs in which the short N-terminal palmitoylation motif of PSD-95 had been replaced with that of GAP43 (+GAP43-N11, Fig. S1 D) or with another type of acylation motif (the N-terminal myristoylation motif of Src, +Src-N20; Fig. S1 E). Furthermore, the antibody did not react with either a short palmitoylated motif from PSD-95 (N13, 1–13 aa) or with palmitoylated 1-SH3 (1–495 aa), which lacked the GuK domain (Fig. 3 F). In contrast, PSD-95 d(PDZ1,2), which lacked the first and second PDZ domains, was detected by PF11 in a palmitoylation-dependent manner (Fig. S1, F and G). The GuK domain (533–699 aa) was necessary for the interaction of palmitoylated PSD-95 d(PDZ1,2) with PF11 (Fig. 3 F and Fig. S1 F). However, the GuK domain alone was not sufficient for binding with palmitoylated PSD-95 (not depicted). When DHHC2 palmitoylating activity was blocked with 2-BP, PSD-95-mCherry detached from the plasma membrane. PF11-GFP was similarly released in the cytoplasm, dissociating from the plasma membrane and from intracellular aggregates of PSD-95-mCherry (Fig. 3, D and F). Specific binding of PF11-GFP to palmitoylated PSD-95 was also observed in immunoprecipitation experiments. More PSD-95-mCherry was coimmunoprecipitated with PF11-GFP when cells overexpressed DHHC2, whereas the binding was prevented in cells pretreated with 2-BP (Fig. 3 E). Binding of PF11-GFP to PSD-95 CS did not occur. Thus, PF11 can serve as an intrabody to track palmitoylated PSD-95 in intact cells.

We next asked whether PF11 is a palmitoylation site-specific or conformation-specific antibody. We coexpressed a series of chimeric or deleted PSD-95-GFP constructs with PF11-mCherry in HEK293T cells (Fig. 3 F and Fig. S1, A–G). A PSD-95 mutant that contained a prenylated and dually palmitoylated motif of paralemmin at the C terminus of PSD-95 CS-GFP (PSD-95 CS-GFP/Para) was localized at the plasma membrane with palmitoylated PSD-95 (not depicted). When DHHC2 palmitoylating activity was blocked with 2-BP, PSD-95-mCherry detached from the plasma membrane. PF11-GFP was similarly released in the cytoplasm, dissociating from the plasma membrane and from intracellular aggregates of PSD-95-mCherry (Fig. 3, D and F). Specific binding of PF11-GFP to palmitoylated PSD-95 was also observed in immunoprecipitation experiments. More PSD-95-mCherry was coimmunoprecipitated with PF11-GFP when cells overexpressed DHHC2, whereas the binding was prevented in cells pretreated with 2-BP (Fig. 3 E). Binding of PF11-GFP to PSD-95 CS did not occur. Thus, PF11 can serve as an intrabody to track palmitoylated PSD-95 in intact cells.

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PF11 as a conformation-specific intrabody for palmitoylated PSD-95. (A–D) In the presence of DHHC2, PSD-95-mCherry and PF11-GFP were colocalized near the plasma membrane and on intracellular vesicular structures in HEK293T cells (B and C, white arrowheads). Treatment with 2-BP (100 µM, 8 h) dissociated PF11-GFP from the plasma membrane (asterisks) and intracellular aggregates of PSD-95-mCherry (D, black arrowheads). Bars, 10 µm (2 µm, magnified in C). (E) Immunoprecipitation (IP) of PF11-GFP from HEK293T cells. IB, immunoblotting. CC7, unrelated recombinant antibody; closed arrow, PF11-GFP; open arrow, CC7-GFP; closed arrowhead, PSD-95-mCherry. (F) PSD-95 constructs to test the specificity of PF11 binding using methods in Fig. 3, B and/or E. GuK, guanylate kinase domain; S, serine residues replacing palmitoyl cysteines. Red chain, palmitoyl; green, prenyl; blue, myristoyl groups. tag, mCherry or GFP. (G) Blockade of palmitoylation with 2-BP (100 µM, 18 h) dispersed synaptic labeling by PF11-GFP. Red, mCherry as a fill-in marker. Arrows, multiple subspine clusters. Bars, 5 µm (2 µm, magnified). (H) PF11-GFP tracked a synaptic increase of palmitoylated PSD-95 upon TTX treatment. Arrowheads, multicluster spines. Bar, 5 µm. (I) Immunoprecipitation of PF11-GFP from hippocampal neurons treated with 2-BP or TTX. Arrow, PF11-GFP. Closed and open arrowheads are as in Fig. 1 A.

These results strongly suggest that palmitoylation induces a change in PSD-95 conformation that can be sensed by PF11 and that PF11 recognizes a nonlinear epitope on the palmitoylated form of PSD-95 as reported for other antibodies (Vielemeyer et al., 2010). Fitting with this, hPF11 antibody was not applicable to Western blot of denatured samples (not depicted).

Next, to investigate the behavior of endogenous palmitoylated PSD-95, we expressed PF11-GFP in cultured hippocampal neurons. When visualized in fixed and in live neurons, PF11-GFP labeling was specifically enriched at discrete clusters in dendritic spines (Fig. 3, G and H; Fig. S1, H and I). PF11 intrabody expression did not inhibit the function of PSD-95 (Fig. S1, J and K) and could therefore be used to follow endogenous palmitoylated PSD-95 in intact neurons. Time-lapse imaging revealed a decrease in postsynaptic PF11-GFP signals over time upon 2-BP treatment (Fig. 3 G), thus visualizing in real time depalmitoylation of synaptic PSD-95. This decrease was not caused by photobleaching of GFP, as activity blockade by TTX under the same illumination conditions actually increased the postsynaptic PF11-GFP signal (Fig. 3 H), fitting with previous findings (Noritake et al., 2009). Additionally, immunoprecipitation of PF11-GFP from transfected neurons showed a decrease of PF11 binding to PSD-95 after 2-BP treatment and an increase after TTX treatment (Fig. 3 I). Taken together, these results indicate that PF11-GFP specifically binds to palmitoylated (and thus conformationally changed) PSD-95 in neurons and dissociates from PSD-95 upon depalmitoylation.

Subsynaptic nanodomains of palmitoylated PSD-95 as building blocks of the PSD

When analyzing 3D rendering of confocal image stacks in live neurons, we occasionally detected multiple small PF11-GFP clusters within a single dendritic spine (Fig. 3, G and H). Stimulated emission depletion (STED) super-resolution microscopy more
Figure 4. **Novel PSD-95 nanodomains as building blocks of postsynaptic membrane regions.** (A and B) Live-cell imaging of PF11-GFP by STED microscopy (green), but not by conventional confocal microscopy (red pseudocolor), efficiently detects multiple subspine clusters (1 to 4 clusters/spine) in neurons. Bars: (A) 1 µm; (B) 500 nm. (C and D) Size of subsynaptic nanodomains. Subsynaptic clusters of PF11-GFP and PSD-95-GFP visualized by live STED imaging were measured across the longest axis. Approximately 200 clusters were randomly selected from 10 live-imaging experiments. FWHM, full width at half maximum. ***, P < 0.001 by Student’s t test. (E) The size of postsynaptic membrane regions (determined by confocal microscopy, red in B, according to
efficiently detected PF11-GFP labeling of these unique subsynaptic structures in live neurons (Fig. 4, A and B). Conventional confocal microscopy did not readily resolve these subsynaptic clusters; rather it visualized them as a single cluster (especially in the single-plane image acquisition mode), even when the same field was acquired by confocal and STED modes (Fig. 4 B and Fig. S2 A). Merged images of STED and confocal observation of PF11-GFP demonstrated that the conventional PSD (here, visualized by confocal PF11-GFP signals) represents, in fact, the apposition of several (up to >4) subsynaptic clusters of palmitoylated PSD-95 (visualized by STED PF11-GFP signals). Interestingly, the size of subsynaptic clusters was relatively uniform, irrespective of the number of clusters per synapse (Fig. 4, A and B). The average diameter of subsynaptic clusters, hereafter termed “subsynaptic nanodomains,” was ~200 nm (STED full width at half maximum [FWHM] 211.0 ± 48.8 nm, n = 232 clusters; Fig. 4 C). Size distribution histogram for PF11-labeled nanodomains was symmetric without apparent longer size tails (Fig. 4 D). Nanodomains in the postsynaptic membrane were separated from each other by narrow gaps (less than ~200 nm, i.e., below the resolution of confocal microscopy; Fig. 4, A and B; Fig. S2 A). Based on these observations, we hypothesized that PSD-95 nanodomains represent subdomains of the PSD and that their number could be a determinant of PSD size. Indeed, there was a good correlation between the number of PF11-labeled nanodomains per synapse and the overall size of a given postsynaptic membrane as determined by confocal PF11-GFP (correlation coefficient R = 0.8952; Fig. 4 E). Furthermore, live-cell STED imaging also revealed that conventional PSD-95-GFP existed in similar subsynaptic clusters within spines (Fig. S2 A), although PSD-95-GFP-labeled subsynaptic clusters were larger (STED FWHM 295.3 ± 78.9 nm, n = 203 clusters) than PF11-labeled (endogenous PSD-95) nanodomains (Fig. 4, C and D).

We next performed dual-color STED imaging (2C-STED) of fixed hippocampal neurons by hPF11 and conventional PSD-95 immunofluorescence (Fig. 4 F). STED imaging of hPF11, but not confocal imaging, resolved subsynaptic nanodomains in fixed neurons, despite the reduced resolution that was mostly likely caused by fixation-induced structure shrinkage (Fig. 4 F and Fig. S2 B). As expected, hPF11 signals overlapped with total PSD-95 signals, which also showed nanodomain organization (Fig. 4 F, top panels). Co-staining with vGlut1 showed that individual nanodomains of the postsynapse faced a single presynaptic terminal (Fig. 4 F, bottom panels). The size of the hPF11-labeled postsynaptic membrane region was defined as the FWHM across the longest points of the STED hPF11 signal (Fig. S2 B) and ranged widely from 150 nm to ~1 μm with a mean diameter of 351.9 ± 148.4 nm (n = 469 synapses; Fig. 4 G). This value was compatible with the PSD size previously reported in an electron microscopic analysis (mean size, ~360 nm; Petersen et al., 2003; Arellano et al., 2007). The size distribution showed the strongest frequency peak at around 200–250 nm and could be fitted with at least two additional Gaussian peaks at ~420 and ~625 nm (Fig. 4 G, red dashed curves). A similar size distribution had been previously described for PSDs in an electron microscopic analysis (Arellano et al., 2007; Swulius et al., 2010). It is conceivable that the PF11-labeled postsynaptic structure may represent the PSD, and that the PSD may be composed by a set of palmitoylated PSD-95–enriched subdomains with diameters of ~200 nm.

Because PSD-95 anchors AMPARs at the postsynapse through interaction with stargazin and related transmembrane AMPAR regulatory proteins (TARPs; Tomita et al., 2003; Elias and Nicoll, 2007), we investigated whether individual postsynaptic nanodomains associate with the endogenous GluA1 AMPAR subunit. Neurons expressing PF11-Venus were live-labeled with an anti-GluA1 antibody targeted to an extracellular epitope, after which PF11-Venus and surface-expressed AMPARs were visualized using live-cell 2C-STED imaging. AMPAR clusters appeared to be associated with distinct nanodomains (Fig. 4 H; also see Fig. 9 A).

Continuous palmitate cycling on PSD-95 nanodomains driven by local palmitoylating activity

We then examined the dynamics of PSD-95 palmitoylation and where in neurons palmitoylation, depalmitoylation, and eventually repalmitoylation of PSD-95 occur. We compared fluorescence recovery rates after photobleaching (FRAP) of PF11-GFP and PSD-95-GFP at individual clusters within spines. In agreement with previous reports (Kuriu et al., 2006; Blanpied et al., 2008), the recovery rate of PSD-95-GFP was very slow (14.5 ± 2.6% recovery at 1 h after bleaching; Fig. 5, A, C, and D; Video 1). In parallel, we performed the FRAP of PF11-GFP. The recovery rate of PF11-GFP clusters was much faster (55.7 ± 8.8% recovery at 1 h after bleaching; Fig. 5, A–D; Video 2), and the recovered PF11-GFP fluorescence appeared within the narrowly focused area at the original locations of the nanodomains in a spine (Fig. 5 A, right magnified images, and Video 2). Part of this recovery can be attributed to dynamics of PF11 binding to its target, while another part may be due to cycling of PSD-95 between palmitoylated and depalmitoylated forms. However, this recovery was completely inhibited by treatment with 2-BP at any time points during the recovery period (Fig. 5, A, C, and D), indicating that it mostly depends on active palmitoylation of PSD-95. In contrast, 2-BP treatment did not affect the recovery of PSD-95-GFP, indicating that arrival of new PSD-95-GFP in the spine does not depend on on-going palmitoylation. Thus, on...
the one hand, the total pool of PSD-95 appeared mostly retained within the same spine, barely being exchanged during a 1-h observation, and on the other hand, the same pool of PSD-95 in a given spine was continuously cycling between (re)palmitoylated and depalmitoylated forms. It is conceivable that local palmitoylating activity at the postsynaptic membrane defines the precise locus of PSD-95 nanodomains. Accordingly, N-myristoylated PSD-95 (Src-N20/PSD-95-GFP), which is irreversibly lipidated and cannot be affected by palmitoyl enzymes, is membrane targeted in neurons but is not efficiently clustered at dendritic spines (Fig. S3 A), as described previously (Craven et al., 1999).

**DHHC2, a plasma membrane-inserted palmitoylating enzyme, is required for postsynaptic PSD-95 nanodomain formation**

Because DHHC2, a major PSD-95 PAT, is located on vesicle structures in dendrites and often in dendritic spines (Noritake et al., 2009), we explored the possibility that DHHC2 would palmitoylate PSD-95 locally on the spine membrane to generate postsynaptic PSD-95 nanodomains and define the site of PSD organization. To detect DHHC2 on the cell surface, a hemagglutinin (HA) tag was inserted in the second luminal loop of the enzyme (DHHC2-lumHA, Fig. 6 A). Cell surface staining with an anti-HA antibody detected DHHC2 expressed at the plasma membrane as clusters in dendritic spines (Fig. 6 B). Cell surface expression of DHHC2 was observed before in PC12 cells (Greaves et al., 2011). In contrast, Golgi-localized DHHC3, another subfamily member of PSD-95 PATs, was not detected on the cell surface. Endogenous DHHC2 was found as discrete small puncta in the hPF11-stained postsynaptic region (at the center of two nanodomains; Fig. 6 C). GFP-DHHC2 was localized mainly on transferrin receptor–positive recycling endosomes in the dendrite (Fig. 6 D, left), as described in PC12 cells (Greaves et al., 2011) and juxtaposed to PSD-95-mCherry clusters in the spines (Fig. 6 D, middle). Live-cell imaging using super-elliptic pHluorin (SEP)–tagged...
To explore whether the surface expression of DHHC2 is necessary for the generation of postsynaptic nanodomains of PSD-95, we generated a DHHC2 construct with an ER-retention signal (EKKNR) at its C terminus (DHHC2 ER; Fig. 6 A). DHHC2, which allows to specifically monitor surface-expressed DHHC2, showed the complete colocalization of surface DHHC2 with PSD-95–mCherry clusters at the postsynaptic membrane (Fig. 6 D, right).
DHHC2 accumulated at the ER, and PSD-95-mCherry was distributed diffusely in the cytoplasm; and that (2) biotin-induced synchronized trafficking of SBP-EGFP-DHHC2 to the plasma membrane caused subsequent specific accumulation of PSD-95 at the same plasma membrane regions (Fig. 7, A [left] and B; Video 3). This effect of DHHC2 on PSD-95 accumulation requires its palmitoylating activity, as PSD-95 accumulation was not observed in the presence of 2-BP (Fig. 7, A [right] and B; Video 4) or when catalytically inactive DHHC2 (CS) was expressed (Fig. 7 B and Video 5). Similarly, forced engagement of DHHC2-lum HA with anti-HA antibody on the cell surface (Fig. S4 B) induced PSD-95 recruitment in COS7 cells and neurons (Fig. S4, C–E). Thus, plasma membrane–targeted DHHC2 catalyzes the local PSD-95 palmitoylation and then specific PSD-95 accumulation at the plasma membrane.

**Plasma membrane insertion of DHHC2 triggers specific PSD-95 accumulation at the plasma membrane**

To show a direct causative relationship between DHHC2 plasma membrane targeting and subsequent PSD-95 accumulation, we took advantage of our recently developed synchronized secretion system (retention using selective hooks [RUSH]; Boncompain et al., 2012) that allowed the efficient synchronized trafficking of DHHC2 to the plasma membrane. As illustrated in Fig. S4 A, streptavidin-binding peptide (SBP)–fused GFP-DHHC2 (SBP-GFP-DHHC2) was hooked at the ER by the interaction with the streptavidin (Str)-fused ER-resident protein Ii. Upon addition of biotin, SBP-GFP-DHHC2 was acutely released from the ER, entered the Golgi apparatus (∼10 min after biotin addition), and got sorted into the secretory pathway to its final destination, the plasma membrane (visible around ∼30 min; Fig. 7 A, top left; Video 3). Using this RUSH system we coexpressed PSD-95-mCherry with SBP-GFP-DHHC2. Time-lapse imaging and its kymograph analysis clearly show that (1) before biotin addition, DHHC2 accumulated at the ER, and PSD-95-mCherry was distributed diffusely in the cytoplasm; and that (2) biotin-induced synchronized trafficking of SBP-EGFP-DHHC2 to the plasma membrane caused subsequent specific accumulation of PSD-95 at the same plasma membrane regions (Fig. 7, A [left] and B; Video 3). This effect of DHHC2 on PSD-95 accumulation requires its palmitoylating activity, as PSD-95 accumulation was not observed in the presence of 2-BP (Fig. 7, A [right] and B; Video 4) or when catalytically inactive DHHC2 (CS) was expressed (Fig. 7 B and Video 5). Similarly, forced engagement of DHHC2-lum HA with anti-HA antibody on the cell surface (Fig. S4 B) induced PSD-95 recruitment in COS7 cells and neurons (Fig. S4, C–E). Thus, plasma membrane–targeted DHHC2 catalyzes the local PSD-95 palmitoylation and then specific PSD-95 accumulation at the plasma membrane.

**Changes in synaptic activity alter the core postsynaptic architecture through the local palmitoylating machinery**

We next asked how altered palmitoyl cycling on postsynaptic PSD-95 affects the postsynaptic structure. We stimulated cultured neurons with 90 mM KCl for 5 min, which activates synaptic glutamate receptors and enhances depalmitoylation of PSD-95 (Kang et al., 2004). Neurons were then stained triply with hPF11, PSD-95, and vGlut1 antibodies. Acute depolarization robustly reduced the fluorescence intensity of hPF11-stained postsynaptic clusters and modestly reduced PSD-95 cluster intensity (Fig. 8, A and B). This reduction in hPF11 intensity was reversed when high K⁺ medium was replaced with basal medium and neurons were incubated for 1 h. Importantly, STED microscopy revealed that upon depolarization, signals of total PSD-95 that overlapped with nanodomains of PF11-labeled postsynapses were reduced, and stronger PSD-95 signals were observed additionally in areas independent of nanodomain locations (Fig. 8 C and Fig. S5 A). Approximately 30% of observed
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DHHC2 dynamically defines postsynaptic nanodomains

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Fig. S5, A and B). Furthermore, 2C-STED imaging using bas-

soon co-staining, a marker protein of the presynaptic active

zone, supports the displacement of some populations of PSD-95,

but not palmitoylated PSD-95, from the synaptic sites by high

PSD-95 signals were >~100 nm away from hPF11-labeled

postsynaptic areas in the high K⁺ condition, whereas they were

overlapping under the basal condition (>95%) were within sub-

resolution distance, gray region in the graph; Fig. 8 D and

Fig. S5, A and B). Furthermore, 2C-STED imaging using bas-

soon co-staining, a marker protein of the presynaptic active

zone, supports the displacement of some populations of PSD-95,

but not palmitoylated PSD-95, from the synaptic sites by high

Figure 8. Changes in synaptic activity remodel postsynaptic PSD-95 nanodomains through local DHHC2 activity. (A and B) Neurons were treated with 90 mM KCl for 5 min and recovered for 60 min in the basal medium (wash out, WO). Neurons were stained triply with hPF11 [green], PSD-95 [red], and vGlut1 [blue] antibodies, and the confocal fluorescence intensities of green and red channels at PSD-95–positive clusters were measured. In total, 170–240 clusters from three neurons were analyzed. ***, P < 0.001 by one-way ANOVA with post-hoc Tukey’s test (B). Bar, 5 µm. (C and D) Neurons treated with high K⁺ were analyzed by 2C-STED imaging of PSD-95 (green pseudocolor) and hPF11 (red pseudocolor), and the distance between the peaks with the highest intensity of hPF11 (arrowheads) and PSD-95 (arrows) clusters was measured (as described in Fig. S5 A). The brightness of magnified images with KCl treatment is enhanced. Gray region in D indicates the subresolution range for STED imaging. In total, 150–200 clusters from 10 neurons (two independent experiments) were analyzed. ***, P < 0.001 by Student’s t test (D). Bars, 500 nm (200 nm, magnified). (E and F) Neurons were cotransfected with miR-DHHC2 and HA-resDHHC2-WT or ER [red], and treated as in (A). The confocal fluorescence intensity of hPF11 [green] overlapped with vGlut1 [not depicted] was measured. In total, 230–420 clusters from 8 neurons (three independent experiments) were analyzed. ***, P < 0.001 by one-way ANOVA with post-hoc Tukey’s test. Bar, 5 µm.
recover after removal of the high K⁺ medium. Thus, postsynaptic PSD-95 clusters are dynamically remodeled on a nanoscale level by synaptic activity–regulated palmitoyl cycling on PSD-95, and spine membrane–inserted DHHC2 ensures this exquisite spatial organization of postsynaptic membrane regions.

We finally investigated whether changes in synaptic activity affect the association of AMPARs with postsynaptic nanodomains. We found that high K⁺–induced depolarization significantly reduced the fluorescence intensity of surface AMPARs on hPF11-labeled postsynaptic clusters by 2C-STED imaging, and that this reduction was reversed upon washing out of high K⁺ medium (Fig. 9, A and B).

Discussion

The exact mechanism involved in the initiation, maintenance, and remodeling of PSD organization remains controversial. Here, we propose a subdomain model for the PSD organization.
Propionibacterium DHHC2 was not able to induce membrane recruitment of PSD-95 to endomembranes. Strikingly, we showed that ER-localized palmitoylation. Thus, the site of protein palmitoylation is not restricted to plasma membrane targeting and local PSD-95 palmitylation. Multiple nanodomains (Fig. 6, E and F). When additional membrane DHHC2 reduces the number of large synapses with more than one nanodomain (Fig. 4, E and G), and that loss of plasma DHHC2-mediated repalmitoylation of the PSD-95 C terminus (Gorleku et al., 2011). was obtained by adding the ER-retention signal (EKKNR) of DHHC6 to its insertion SEP cDNA between 198T and 199N of DHHC2. DHHC2-ER and 204K of DHHC3, respectively. DHHC2-GFP (Noritake et al., 2004). To generate HA-tag) were isolated by RT-PCR from mouse brain total RNAs and constructed by replacing the N-terminal palmitoylation motif of PSD-95 (aa 1–13; El-Husseini et al., 2000a); d(PDZ1,2) lacking aa 65–247; d(PDZ1,2; EMD Millipore). In brief, the tobacco etch virus (TEV) protease recognition site (ENLYFQ), a (Gly) linker, and a biotin acceptor peptide (Propionibacterium shermanii transcarboxylase domain [PSTD]), GenBank/EMBL/DDJB accession no. AJ532501) were tandemly added to the C terminus of PSD-95-GFP. The single-chain fragment V5 (scFvA), PF11 and CC7 were subcloned into pEGFP-N3 or pmCherry-N3 and used for expression in HEK293T cells. PF11-GFP and CC7-GFP were then subcloned into pCAGGS. GAP43 (aa 1–11; dually palmitoylated) or Src (aa 1–20; myristoylated), were subcloned into pGW1-GFP vector. pGW1-PSD-95 CS-GFP was generated using site-directed mutagenesis. PSD-95 1–SH3 [aa 1–493]; N13 [aa 1–13]; ELHusseini et al., 2000a); d(PDZ1,2) lacking aa 65–247; d(PDZ1,2; GUK) lacking aa 533–699 from PSD-95 d(PDZ1,2); and GUK [aa 533–699] were generated by PCR and subcloned into pGWI-GFP vector. pGWI-PSD-95 CS-GFP/Para was generated by appending a synthetic DNA encoding the prenylation motif of paraclenmin (DKMKHRKSKSCIM in a residue, affininity purified. The following reagents were used: 2-bromohexadecanoic acid (2-bromopalmitate, 2-8P; Fluka), tetrodotoxin (TTX; Nacalai Tesque), picotroxin (Sigma-Aldrich), t-biotin (Sigma-Aldrich), and anisomycin (EMD Millipore). Plasmid constructions To obtain the antigen for antibody screening, pGW1-PSD-95-GFP-TEV-Gly-PSTCD was generated by a standard PCR method. The PSTCD tag (McCormick et al., 2006) was extracted from the pPinpoint plasmid (Promega). In brief, the tobacco etch virus (TEV) protease recognition site (ENLYFQ), a (Gly) linker, and a biotin acceptor peptide (Propionibacterium shermanii transcarboxylase domain [PSTD], GenBank/EMBL/DDJB accession no. AJ532501) were tandemly added to the C terminus of PSD-95-GFP. The single-chain fragment V5 (scFvA), PF11 and CC7, were subcloned into pEGFP-N3 or pmCherry-N3 and used for expression in HEK293T cells. PF11-GFP and CC7-GFP were then subcloned into pCAGGS for neuronal expression. Rat PSD-95 [aa 1–724] and PSD-93[157] cDNAs were subcloned into pGW1-GFP vector. pGWI-PSD-95 CS-GFP was generated using site-directed mutagenesis. PSD-95 1–SH3 [aa 1–493]; N13 [aa 1–13]; ELHusseini et al., 2000a); d(PDZ1,2) lacking aa 65–247; d(PDZ1,2; GUK) lacking aa 533–699 from PSD-95 d(PDZ1,2); and GUK [aa 533–699] were generated by PCR and subcloned into pGWI-GFP vector. pGWI-PSD-95 CS-GFP/Para was generated by appending a synthetic DNA encoding the prenylation motif of paraclenmin (DKMKHRKSKSCIM in aa) to the C terminus of PSD-95 CS-GFP (ELHusseini Ael et al., 2002). pGW1-GAP43 N11/PSD-95-GFP and pGW1-Src N20/PSD-95-GFP were constructed by replacing the N-terminal palmitoylation motif of PSD-95 [aa 1–12 or aa 1–20] with the synthetic DNA encoding a fatty acylation motif of GAP43 [aa 1–11; doubly palmitoylated] or Src [aa 1–20; myristoylated], respectively. Mouse DHHC2 and DHHC3 (with N-terminal, cytoplasmic HA-tag) were isolated by RTPCR from mouse brain total RNAs and subcloned into pEF-Bos-HA vector (Fukata et al., 2004). To generate DHHC2-WHA and DHHC3-WHA in which the HA epitope (YPYDVPDYA) is placed in the second luminaly oriented loop, HA was inserted with a Gly–Gly linker between 198T and 199N of DHHC2 and between 203T and 204K of DHHC3, respectively. DHHC2-WHA and DHHC3-WHA were affinity purified by inserting SEP cDNA between 198T and 199N of DHHC2. DHHC2-ER was obtained by adding the ER-retention signal (EKKNR) of DHHC6 to its C terminus (Gorleku et al., 2011).
PSD-95 was knocked down by transfecting pLox3.7-shPSD-95 together with a GFP reporter (American Type Culture Collection; Elias et al., 2006). For molecular replacement experiments, shRNA-resistant PSD-95 constructs (resPSD-95 WT, CS) that have four different nucleotides in the target sequences were generated using site-directed mutagenesis (S-TCA-CAATAATGCCCCAGTAGTA3′; [changed nucleotides are underlined]; Noritake et al., 2009). Knockdown of DHHC2 was performed using the miRNA system with EmGFP or mCherry promoters (Invitrogen), and miRNA-resistant DHHC2 constructs (resDH2) were used for molecular replacement experiments as described previously (Noritake et al., 2009). In Fig. 6, E and F, PF1 1-GFP was replaced with EmGFP reporter for the co-cistronic expression of PF1 1-GFP and mirDHHC2.

For RUSH assay, steptavidin-fused li and SFB-fused GFP-DHHC2 were used for the ER hook and the reporter, respectively. These genes were expressed under a CMV promoter and were separated by a synthetic intron and an internal ribosome entry site (Boncompain et al., 2012).

R.Y. Tsien (University of California, San Diego, La Jolla, CA), G. Miesenboeck (University of Oxford, Oxford, England, UK), and V.V. Verkhovsky (Albert Einstein College of Medicine, Bronx, NY) provided us with cDNAs encoding mCherry (Shaner et al., 2005), SEP (Miesenbéc et al., 1998), and transferrin receptor (Subach et al., 2009), respectively. PSD-95 deletion and chimeric constructs were provided by D.S. Bredt (Johnson & Johnson, San Diego, CA). All PCR products were analyzed by DNA sequencing (Functional Genomics Facility, NIBB, Aichi, Japan).

**Purification of palmitoylated PSD-95**

To purify full-length palmitoylated PSD-95 as the antigen, PSD-95-GFP was fused to a PSTCD tag (see Plasmid constructions), which undergoes biotinylation by endogenous enzymes in mammalian cells (Lorenzen et al., 2004). PSD-95-GFP-TEV-PSTCD and DHHC15, which stoichiometrically palmitoylate PSD-95 (Fukata et al., 2004), were coexpressed in HEK293T cells for 24 h. The cells were then lysed with buffer A (25 mM Tris·HCl, pH 7.5, 2 mM EDTA, 100 mM NaCl, 0.2% Triton X-100, and 0.05 µg/ml PMSF). The lysates were spun at 20,000 × g for 30 min at 4°C and the supernatants were mixed with streptavidin-coated magnetic beads (SA Dynabeads M-280; Invitrogen) for 2 h at 4°C. The beads were washed with buffer B (25 mM Tris·HCl, pH 7.5, 2 mM EDTA, 100 mM NaCl, 0.1% Triton X-100, and 1 mM DTT) and used for antibody phage display screening.

**Antibody phage display screening**

The screening was performed using the Griffin.1 library of scFv, which is a random combination of human repertoires of heavy and light chains that was provided by G. Winter (MRC, Cambridge, England, UK; www.lifesciences.sourcebioscience.com; Nizak et al., 2003a,b; Dimitrov et al., 2006). The screening was performed using the Griffin.1 library of scFv, which is a random combination of human repertoires of heavy and light chains that was provided by G. Winter (MRC, Cambridge, England, UK; www.lifesciences.sourcebioscience.com; Nizak et al., 2003a,b; Dimitrov et al., 2006).

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was performed at 17–21 DIV with the same equipment. 3D image stacks were taken on dendritic regions of a neuron and then projected to 2D images using maximal intensity.

For RUSH analysis (Boncompain et al., 2012), HEK293T cells were cotransfected with a RUSH plasmid containing ER-Hook (streptavidin-II) and SBF-GFP-DHHC2 together with pGW1-PSD-95-mCherry. At time 0, 40 µM O-biotin and 25 µM aminoacylase were added and xyt scans were performed every minute for 90 min with a laser-based auto-focusing system (adaptively focus control) at 37°C in a 5% CO₂ chamber with the confocal mode of a confocal system (TCS SP5 II; Leica) with the HyD Detectors. Objective lens used was an HCX PL APO 63×/1.40 NA oil. Kymographs were produced using MetaMorph software version 7.7 (Molecular Devices).

For FRAP analysis, neurons were transfected with PF11-GFP or PSD-95-GFP, and the dendritic region indicated by a white rectangle was bleached with a 488 nm laser. We acquired xyt stacks at a spacing of 0.5 µm (5–7 sections) every 2 min for 1 h, with or without 2-BP treatment at 37°C in a 5% CO₂ chamber with the confocal mode of a Leica TCS SP5 II with the HyD Detectors. Images were reconstructed using maximum intensity projections; individual clusters in the spine were tracked at each frame, and their average intensities were plotted in the graph. To exclude the effect of rapid diffusion of GFP probes from soma to the dendrite (shunt and spine), background fluorescence intensities in the bleached dendritic shaft were subtracted from those in the individual cluster at every time point. Image analysis was performed with LAS-AF software (Leica). Our FRAP data fit with the data of rapid palmitate turnover on PSD-95 measured by the pulse-chase method (El-Husseini Ael et al., 2002), but does not with the data of slow turnover measured by the acyl-biotinylation exchange method (Thomas et al., 2012). Although these biochemical methods showed different extents of palmitate turnover on PSD-95 (El-Husseini Ael et al., 2002; Kang et al., 2008; Noritake et al., 2009; Thomas et al., 2012), our FRAP analysis with PF11-GFP could allow us to sensitively detect rapid palmitate turnover on PSD-95 at the level of individual spines.

For live STED imaging of PF11-GFP or PSD-95-GFP, neurons transfected as above were observed at 23°C in a 5% CO₂ culture chamber. To directly compare STED with confocal imaging, the same optical fields were acquired by the STED mode (TCS SP5 II with the HyD detectors). Images were reconstructed using maximum intensity projection across the longest axis of randomly selected sub-synaptic clusters using LAS-AF software (Leica).

To examine the relationship between PF11-Venus nanodomains and surface GluA1 (Fig. 4 H), surface GluA1 was “live” labeled with an antibody to an extracellular epitope of GluA1 by incubating neurons for 15 min at 37°C. After washing with neurobasal medium, neurons were labeled by an ATTO425-conjugated secondary antibody for 15 min. After neurons were washed with buffer C, living neurons were quickly observed at 458 and 514 nm excitation at room temperature by the STED mode.

Immunohistochemistry

An adult mouse brain was freshly frozen and embedded in the OCT compound. Then, the frozen sections (7 µm in thickness) were cut on a cryostat (CM1950; Leica) and fixed with acetone for 20 min on ice (paraformaldehyde fixation did not work for hPF11). Fixed sections were rehydrated and blocked for 1 h at room temperature in PBS containing 10% donkey and goat serum, and were incubated in the mixture of hPF11, PSD-95, and xGlu antibodies overnight at 4°C, followed by Dylight488 anti-human IgG Fa, Cy3 anti-mouse IgG, and Alexa Fluor 648 anti–guinea pig IgG secondary antibodies, respectively. Fluorescent images were acquired with a confocal laser scanning microscopy system (TCS SP5 II; Leica) equipped with an HCX PL APO 63×/1.40 oil immersion objective lens. To examine whether treatment with 2-BP and HA (which removes palmitate from PSD-95) abolishes staining by hPF11, we treated fixed brain sections or cultured neurons with NH2-HA on the cell surface. For paired sample comparisons, Student’s t tests were used. For multiple test objects, one-way ANOVA with posthoc Tukey’s tests were used. Error bars denote the standard deviation (SD). Box-and-whisker plots are shown to identify the median, 25th, and 75th percentiles, as well as the extremes.

Online supplemental materials

Fig. S1 shows that PF11 intrabody recognizes the conformation of palmitoylated PSD-95 and does not affect the PSD-95 function. Fig. S2 shows postsynaptic nanodomains visualized by PF11-GFP, PSD-95-GFP, and hPF11. Fig. S3 shows that palmitoylation, but not myristoylation, is necessary for the postsynaptic clustering of PSD-95. Fig. S4 shows the schematic diagram of RUSH and cell surface engagement assays. Fig. S5 shows that synaptic activation causes intra-spine delocalization of depalmitoylated PSD-95 and reduces the size of the postsynaptic membrane region. Video 1 shows that the FRAP of PSD-95-GFP is very slow. Video 2 shows that the FRAP of PF11-GFP is much faster. Video 3 shows that acutely induced plasma membrane insertion of DHHC2 triggers accumulation of PSD-95 at the plasma membrane. Video 4 shows that the treatment with 2-BP blocks DHHC2-induced PSD-95 accumulation at the plasma membrane. Video 5 shows that catalytically inactive DHHC2 (CS) does not accumulate PSD-95 at the plasma membrane. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201302071/DC1.

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