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CYLD, a deubiquitinase specific for lysine63-linked polyubiquitins, accumulates at the postsynaptic density in an activity-dependent manner

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ABSTRACT

Polyubiquitin chains on proteins flag them for distinct fates depending on the type of polyubiquitin linkage. While lysine48-linked polyubiquitination directs proteins to proteasomal degradation, lysine63-linked polyubiquitination promotes different protein trafficking and is involved in autophagy. Here we show that postsynaptic density (PSD) fractions from adult rat brain contain deubiquitinase activity that targets both lysine48 and lysine63-linked polyubiquitins. Comparison of PSD fractions with parent subcellular fractions by Western immunoblotting reveals that CYLD, a deubiquitinase specific for lysine63-linked polyubiquitins, is highly enriched in the PSD fraction. Electron microscopic examination of hippocampal neurons in culture under basal conditions shows immunogold label for CYLD at the PSD complex in approximately one in four synapses. Following depolarization by exposure to high K+, the proportion of CYLD-labeled PSDs as well as the labeling intensity of CYLD at the PSD increased by more than eighty percent, indicating that neuronal activity promotes accumulation of CYLD at the PSD. An increase in postsynaptic CYLD following activity would promote removal of lysine63-polyubiquitins from PSD proteins and thus could regulate their trafficking and prevent their autophagic degradation.

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1. Introduction

During the last decade, ubiquitination of synaptic proteins received increasing attention [1–8]. Many of these studies focused on ubiquitination as a tag for proteasomal degradation. The ubiquitin proteasome system (UPS) is responsible for the degradation of the main postsynaptic density (PSD) scaffolds PSD95 [2], Shank and GKAP [1,6] and may promote endocytosis of AMPA receptors [2–4]. Degradation of synaptic proteins by the UPS is thought to be involved in aspects of synaptic plasticity [9].

While attachment of lysine48-linked polyubiquitin chains directs proteins to proteasomal breakdown, attachment of another type of ubiquitin chain, lysine63-linked polyubiquitin, promotes different trafficking of proteins and is involved in diverse functions such as DNA repair, endocytosis, NFkB signaling and most notably in the formation and autophagic clearance of protein aggregates [10,11]. Loss or suppression of autophagy has been demonstrated to promote neurodegeneration [12,13], Thus an impairment of autophagy, in addition to, or instead of, an impairment of proteasomal degradation may be a factor in the development of certain neurodegenerative diseases.

CYLD, originally identified as cylindromatosis tumor suppressor gene, encodes a deubiquitinase specific for lysine63-linked polyubiquitin chains [14]. It is expressed in high levels in the brain [15], although, so far, studies focusing on CYLD in brain have been lacking. A study on p62, a CYLD-binding protein, implied that inhibition of CYLD leads to accumulation of proteins with lysine63-linked polyubiquitination in brains of $p62^{-/-}$ mice [16].

Mass spectrometric analysis of affinity-purified PSD preparations from rat forebrain revealed CYLD to be a prominent protein in affinity purified PSD fractions [17]. Here we investigate the presence of CYLD at PSDs by immunoblotting and immunoEM, and test whether there are changes in PSD-associated CYLD levels following synaptic activity.

2. Materials and methods

2.1. Materials

Antibodies to CYLD:rabbit polyclonal (1:250 or 1:500 for Westerns, 1:100 for EM) from Sigma (SAB4200060) and mouse monoclonal (E-4) from Santa Cruz (sc-74434, 1:500). Antibody to PSD-

Abbreviations: CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II; PSD, postsynaptic density; UPS, ubiquitin proteasome system.

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95 (1:5000):custom made rabbit polyclonal to residues 290–307 by New England Peptide (Gardener, MA). Antibody to alpha-CaMKII mouse monoclonal (clone 6G9, 1:500) Antibody to ubiquitin:mouse monoclonal (clone Ubi-1, aka 042691GS) from Millipore (MAB150).

Di-ubiquitins from Boston Biochem (Cambridge, MA) and polyubiquitins from Enzo Life Sciences (Farmingdale, NY). CYLD lysate (lysate from HEKT 293 cells overexpressing CYLD- transient overexpression of CYLD transcript variant 2) and control cell lysate (same type of cells transfected with empty vector) from Novus Biologicals (Littleton, CO).

The protocols for obtaining brains for subcellular fractionation and hippocampal cultures were approved by NIH Animal Use and Care Committee and conformed to NIH guidelines.

Subcellular fractionation from rat cerebral cortices was carried out as described previously [18]. Brains from adult Sprague Dawley rats were collected and frozen in liquid nitrogen within 2 min of decapitation by Pel-Freeze Biologicals (Rogers, AR). Frozen brains were thawed for 1 min in 0.32 M sucrose at 37 °C and were immediately dissected and homogenized. For rapidly processed PSD preparations, cerebral cortices were dissected out from adult Sprague Dawley rats and homogenized within an average of 1.5 min after decapitation.

2.2. Electrophoresis and immunoblotting

Samples were separated by SDS–PAGE on 4–15% gradient Tris-HCl gels from BioRAD or 4–15% gradient Bis-Tris gels from Life Technologies and transferred to nitrocellulose membranes, blocked, incubated with specified primary antibodies and then with horseradish peroxidase-conjugated secondary antibodies (1:50,000 dilution), and the signal was finally visualized by chemiluminescence (SuperSignal West Pico, Thermo Scientific).

2.3. Deubiquitination assay

Di- or polyubiquitins (0.25 μg in 1 mg/ml BSA) were incubated at 37 °C with PSD fractions (25 μg) in medium containing 0.5 mM EDTA, 0.5 mM EGTA, 100 mM HEPES pH 7.4 in a final volume of 25 μl. Reactions were terminated by the addition of SDS-containing electrophoresis sample buffer. In controls, enzymes were heat-inactivated by 2 min incubation of the PSD fraction at 99 °C.

2.4. Preparation and treatment of dissociated hippocampal cultures

Hippocampal cells from 21-day embryonic Sprague–Dawley rats were dissociated and grown on a glial cell layer as described previously [19] for 19–21 days. Cell cultures were treated as described previously [20]. Control incubation medium contained 124 mM NaCl, 2 mM KCl, 1.24 mM KH_2PO_4, 1.3 mM $MgCl_2$, 2.5 mM $CaCl_2$, 30 mM glucose in 25 mM HEPES at pH 7.4. High K+ medium contained 90 mM KCl with the NaCl concentration reduced accordingly to preserve isotonicity.

2.5. Pre-embedding immunogold-labeling and electron microscopy

After treatment, neuronal cultures were processed for pre-embedding immunogold-labeling as described previously [20]. Briefly, cultures were fixed in 4% paraformaldehyde (EMS, Hatfield, PA) in PBS for 30–45 min at room temperature, permeabilized, incubated with primary and secondary antibodies (Nanogold, Nanoprobes, Yaphank, NY) for 1–1.5 h, then fixed with 2% glutaraldehyde in PBS, silver enhanced (HQ kit, Nanoprobes), and processed for electron microscopy. Only parallel samples from the same experiment were directly compared because the overall labeling sensitivity may differ between experiments.

2.6. Morphometry and statistical analysis

Excitatory synapses are identified by structural characteristics of clustered synaptic vesicles in the presynaptic terminal, the uniform 20 nm separation of the pre- and postsynaptic membrane, and the postsynaptic density (PSD) of dense material underneath the postsynaptic membrane [21]. The PSD complex was defined as the postsynaptic specialization that comprises the electron dense PSD core and the contiguous network [20,22]. The measurement area of the PSD complex was marked by the postsynaptic membrane, a parallel dashed line drawn at 120 nm to the postsynaptic membrane, and two vertical lines to demarcate the area (Fig. 4, bottom right panel). The distance of this measurement border from the PSD complex was set at 120 nm based on previous studies [20,23]. CYLD labels appear as individual black grains in the PSD complex, and labeling intensity was quantified as number of labels per μm length of the postsynaptic membrane.

Every synaptic profile encountered was scored for CYLD antibody labeling on the microscope as negative (0–2 grains) or positive (3 or more grains), and the percentage of CYLD-labeled PSDs was calculated for each sample. Every cross-sectioned, CYLD-positive PSD was photographed with a CCD camera (XR-100 from AMT, Danvers, MA, USA) for the calculation of labeling intensity. Statistical analysis (KaleidaGraph, Synergy Software) was carried out by Student's t test with confidence levels set at $P < 0.01$ unless otherwise indicated.

3. Results

3.1. PSD fractions from rat brains contain deubiquitinase activity

Incubation of multi-ubiquitin chains (Diubiquitins or a mixture of polyubiquitins of differing chain lengths) linked through either lysine48 or lysine63 with PSD fractions, leads to their time-dependent breakdown (Fig. 1). On the other hand, no loss of multi-ubiquitins is observed up to 60 min in control samples when PSD fractions are heat-inactivated prior the incubation. These results indicate the presence in isolated PSDs of deubiquitinases that target lysine48- as well as those that target lysine63-linked multi-ubiquitin chains. Biochemical and immunoEM experiments were carried out to test whether CYLD is one of the deubiquitinases integral to the PSD.

3.2. CYLD is highly enriched in the PSD fraction

Two antibodies raised against epitopes representing non-overlapping sequences of CYLD were used to probe the distribution of the protein in subcellular fractions from brain. The specificity of the antibodies was checked using lysates from HEK 293 cells overexpressing CYLD (Fig. 2A). Both antibodies recognize a band around 110 KDa in PSD fractions, and a band of slightly lower mobility in lysates from cells overexpressing CYLD but not in control lysates. The lower mobility of the band in lysates is due to a DDK tag.

Comparison of subcellular fractions from rat brain in western blots probed with these two CYLD antibodies show a drastic enrichment of the protein in the PSD fraction compared to parent fractions (Fig. 2B). The enrichment of CYLD in the PSD fraction is comparable to that of PSD-95, a marker for PSDs.

3.3. CYLD is less prevalent in PSD fractions from rapidly processed brains

It is known that the relative amounts of CaMKII in the PSD fraction depend on the speed of processing of brains between decapitation and homogenization [24,25]. Presumably, CaMKII