

# A proteasome-sensitive connection between PSD-95 and GluR1 endocytosis

Baris Bingol, Erin M. Schuman\*

*California Institute of Technology, HHMI, Division of Biology, MC 114-96, Pasadena, CA, 91125, USA*

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## Abstract

Synaptic transmission at excitatory synapses can be regulated by changing the number of synaptic glutamate receptors (GluRs) through endocytosis and exocytosis. The endocytosis of GluRs has recently been shown to require the activity of the ubiquitin–proteasome system (UPS): proteasome inhibitors or dominant negative forms of ubiquitin block the ligand-stimulated internalization of GluRs. We have examined whether PSD-95 is a potential target of the UPS. Following neurotransmitter stimulation, PSD-95 levels are negatively correlated with the magnitude of internalized GluR1 in individual neurons. Neurotransmitter stimulation also results in a proteasome-dependent decrease in dendritic PSD-95. Consistent with the idea that PSD-95 degradation is important for GluR internalization, overexpression of PSD-95 can inhibit neurotransmitter-stimulated GluR1 endocytosis. If PSD-95 is a direct target for proteasomal degradation, then the polyubiquitination of PSD-95 is expected. Using experimental conditions that favor the detection of polyubiquitination, however, no ubiquitination of PSD-95 was detected. It is possible that the polyubiquitination of PSD-95 is short-lived and thus difficult to detect. Alternatively, the regulation of PSD-95 levels by the proteasome important for ligand-stimulated GluR endocytosis may be accomplished via an intermediate protein.

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**Keywords:** AMPA receptor; Ubiquitin–proteasome pathway

## 1. Introduction

Synaptic transmission at excitatory synapses can be regulated by changing the number of synaptic glutamate receptors through endocytosis and exocytosis (Carroll et al., 1999; Luscher et al., 1999; Ehlers, 2000; Lin et al., 2000). Like other ligand-gated receptors, AMPA-type glutamate receptors (GluRs) undergo both constitutive and stimulated recycling and trafficking.

The specific stimuli that lead to GluR endocytosis have been characterized, as has the fate of internalized receptors (Carroll et al., 1999; Beattie et al., 2000; Ehlers, 2000; Lin et al., 2000; Man et al., 2000; Snyder et al., 2001; Xiao et al., 2001; Zhou et al., 2001). Less is known, however, about the downstream mechanisms that lead to GluR endocytosis. It has been recently

shown that agonist-induced changes in GluR conformation lead to dissociation of GluR from its intracellular partners, which may lead to endocytosis (Tomita et al., 2004). This view assumes a “ligand binding” model where the occupancy of the glutamate binding site of GluR initiates the endocytosis process. But agonists of other receptors are able to induce GluR endocytosis, which suggests molecular convergence on specific intracellular signaling pathways that regulate GluR endocytosis. Likely targets of these signaling pathways are the GluR-interacting proteins and other proteins present in the postsynaptic density that have been implicated in GluR trafficking.

There are many potential mechanisms by which GluR-interacting proteins could regulate the GluR trafficking. For example, they could stabilize the receptor at the surface (e.g. the actions of GRIP) (Osten et al., 2000), or they could modify exocytosis or endocytosis processes directly or indirectly by changing the ability of the GluR to access the endocytosis/

\* Corresponding author. Tel.: +1-626-395-8390; fax: +1-626-568-0631.

E-mail address: [schumane@its.caltech.edu](mailto:schumane@its.caltech.edu) (E.M. Schuman).

exocytosis machinery (e.g. AP2) (Lee et al., 2002). GluR-interacting proteins that regulate the trafficking of the receptor might be predicted to show changes themselves in response to the same agonists that alter GluR levels at the membrane.

One pathway that regulates GluR endocytosis is the ubiquitin–proteasome system (UPS) (Burbea et al., 2002; Patrick et al., 2003; Colledge et al., 2003). The UPS is one of the primary pathways that cells use to degrade proteins (Smalle and Vierstra, 2004). This pathway includes enzymes that target particular protein(s) to be degraded by attaching polyubiquitin chains. The polyubiquitin chain is then recognized by an enzymatic complex called the proteasome. Both polyubiquitination (Patrick et al., 2003) and proteasome activity (Colledge et al., 2003; Patrick et al., 2003) are required for GluR internalization. As the GluR is primarily degraded by the lysosome (Ehlers, 2000), it is unlikely that the receptor itself serves as the critical proteasomal target, the degradation of which must precede endocytosis. Thus, it is likely that GluR endocytosis is regulated by the targeted degradation of an GluR-interacting protein(s), leading to the destabilization of the receptor and its subsequent endocytosis.

One of the proteins that regulates GluR trafficking is PSD-95 (Chen et al., 2000; El-Husseini Ael et al., 2002; Schnell et al., 2002; Beique and Andrade, 2003). PSD-95 is a major scaffolding protein of the post-synaptic density (Cho et al., 1992). It interacts with GluRs indirectly through another surface protein, called stargazin (Chen et al., 2000). PSD-95 regulates the synaptic clustering of GluRs: when PSD-95 is overexpressed, the synaptic GluR amount increases (El-Husseini et al., 2000), whereas when PSD-95 clusters are dispersed (El-Husseini Ael et al., 2002), there is a declustering of GluRs.

In this paper, we show that the magnitude of AMPA-stimulated GluR endocytosis is negatively correlated with the amount of PSD-95 in dendrites. We also show that the level of PSD-95 in neurons is regulated by AMPA stimulation and proteasome activity. Although these observations suggest that PSD-95 levels are directly regulated by proteasomal activity, we were unable to show that PSD-95 undergoes polyubiquitination (e.g. Colledge et al., 2003), as would be expected if PSD-95 is indeed a target of the UPS.

## 2. Methods

### 2.1. Antibodies

The following antibodies were used as indicated: for immunocytochemistry: PSD-95 6G6 (ABR) and GluR1

(Oncogene). For PSD-95 immunoprecipitation, either rabbit polyclonal (gift of Mary Kennedy), Upstate mouse monoclonal K28/43 or PSD-95 6G6 (ABR); for ubiquitin immunoprecipitation, FK2 mouse monoclonal; for PSD-95 immunoblotting, rabbit polyclonal; for ubiquitin immunoblotting, either DAKO rabbit polyclonal, FK2 mouse monoclonal or BD Pharmingen mouse monoclonal 6C1.17; for Shank immunostaining, rabbit polyclonal (gift of Eujoon Kim); for actin immunostaining, Sigma mouse monoclonal AC-40.

### 2.2. Immunocytochemistry

Dissociated postnatal (P1–2) rat hippocampal neuron cultures prepared as previously described, plated at a density of 230–460 mm<sup>2</sup> (Patrick et al., 2003). Two to three weeks old cultures were used for all the experiments. Receptor internalization assays have been performed as described previously (Patrick et al., 2003). Briefly, neurons were kept in the presence or absence of proteasome inhibitor MG132 (50 μM, Peptide International) for 20 min along with GluR1 antibody and they were stimulated with AMPA (100 μM, 20 min). Following acid stripping and fixation, neurons (Patrick et al., 2003) were labeled with PSD-95. Internalized receptors and PSD-95 were visualized by Alexa-568 rabbit and Alexa-488 mouse secondary antibodies (Molecular Probes), respectively. For PSD-95 staining only, live labeling, acid stripping and Alexa-568 rabbit secondary steps were omitted.

### 2.3. Transfection

In PSD-95 GFP overexpression experiments, neurons were transfected with PSD-95-GFP (a gift of Morgan Sheng) or GFP constructs by Lipofectamine 2000 (Invitrogen). GFP images were acquired before AMPA stimulation. Then, neurons were processed to label internalized receptors as described above.

### 2.4. Image acquisition and analysis

Images shown in Figs. 1 and 2 were acquired via confocal microscopy (Olympus, with Fluoview acquisition software) with a 60X oil objective lens. Shown are the compressed z stack images. Images shown in Fig. 3 were acquired with Olympus microscope (40X immersion objective) with a Hamamatsu CCD camera. All images were taken from similar focal planes. Images were acquired with Image Pro-Plus acquisition software and analyzed on NIH Image J. In both Figs. 1 and 2, internalized receptor and PSD-95 puncta from straightened dendrites were thresholded in such a way to maximize the particle number. Threshold used for

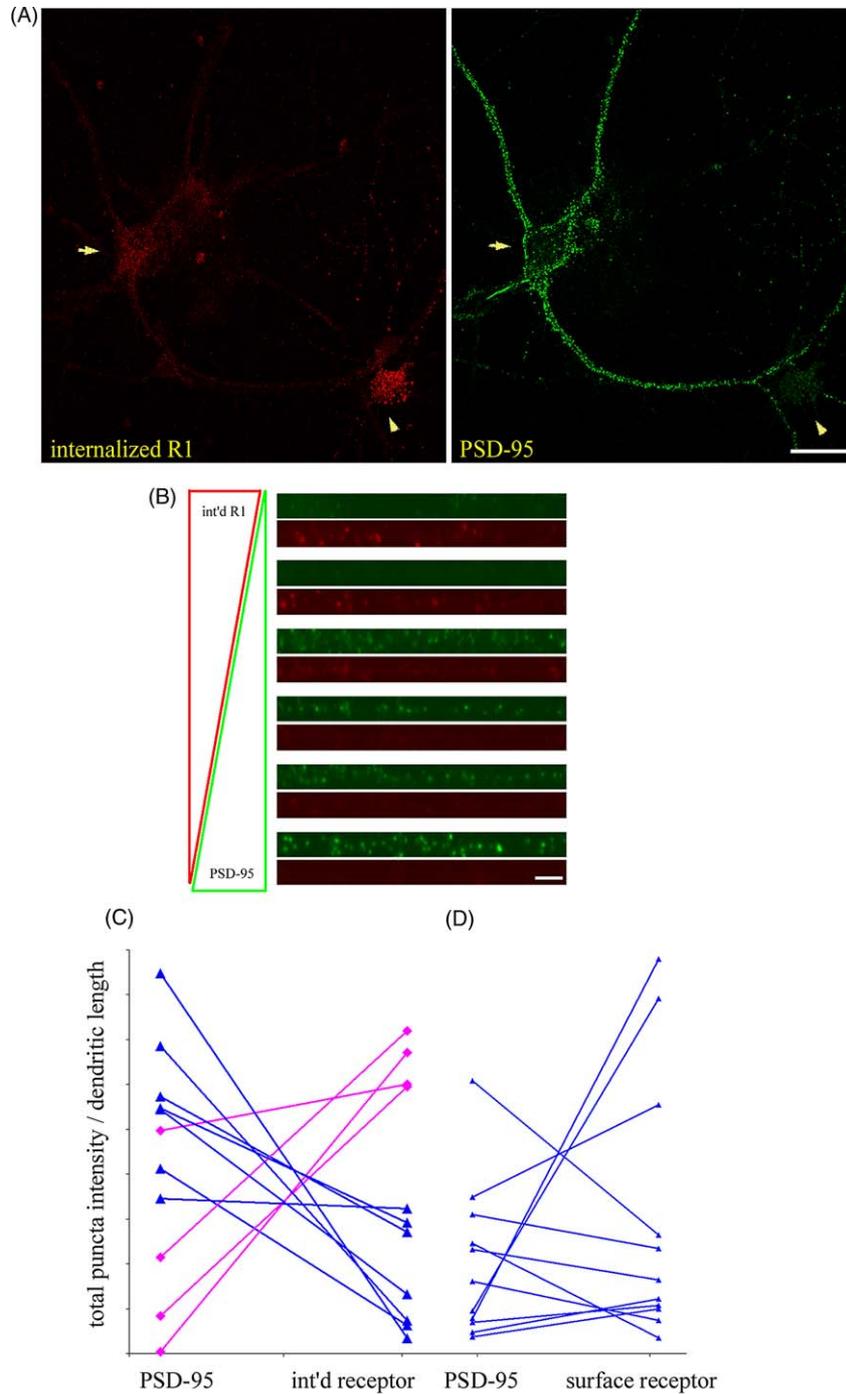


Fig. 1. Inverse correlation between internalized GluR and PSD-95. Both the internalized GluR1 pool and PSD-95 were visualized in individual neurons and dendrites following AMPA stimulation. (A) Neurons that responded robustly to AMPA have more internal GluR1 (red) and less PSD-95 (green) puncta (arrowheads) whereas neurons that did not respond to AMPA have diffuse internal GluR1 staining and more PSD-95 puncta (arrows). Internal GluR1 is visualized using the acid stripping technique (see Methods). Scale bar is 10  $\mu\text{m}$ . (B) Examples of imaged dendrites in which there is an inverse correlation between the amount of internalized receptor (red) and PSD-95 levels (green). Scale bar is 3  $\mu\text{m}$ . (C) Quantification of the data shown in B. Internalized receptor levels and the corresponding PSD-95 level in each dendrite are plotted as the total intensity of signal per dendritic length ( $n = 11$ ,  $r^2 = -0.62$ ,  $p < 0.01$ ). (D) Absence of correlation between surface GluR1 and PSD-95. Surface receptor levels and the corresponding PSD-95 level in each dendrite are plotted as the total intensity of signal per dendritic length ( $n = 11$ ,  $r^2 = 0.02$ ,  $p > 0.5$ ).

individual images were not significantly different between conditions. The thresholded puncta signal was used to quantify the number and total intensity of

puncta per dendritic length. In Fig. 3, internalized receptor signal is quantified from the cell bodies as the fluorescence intensity per unit area.

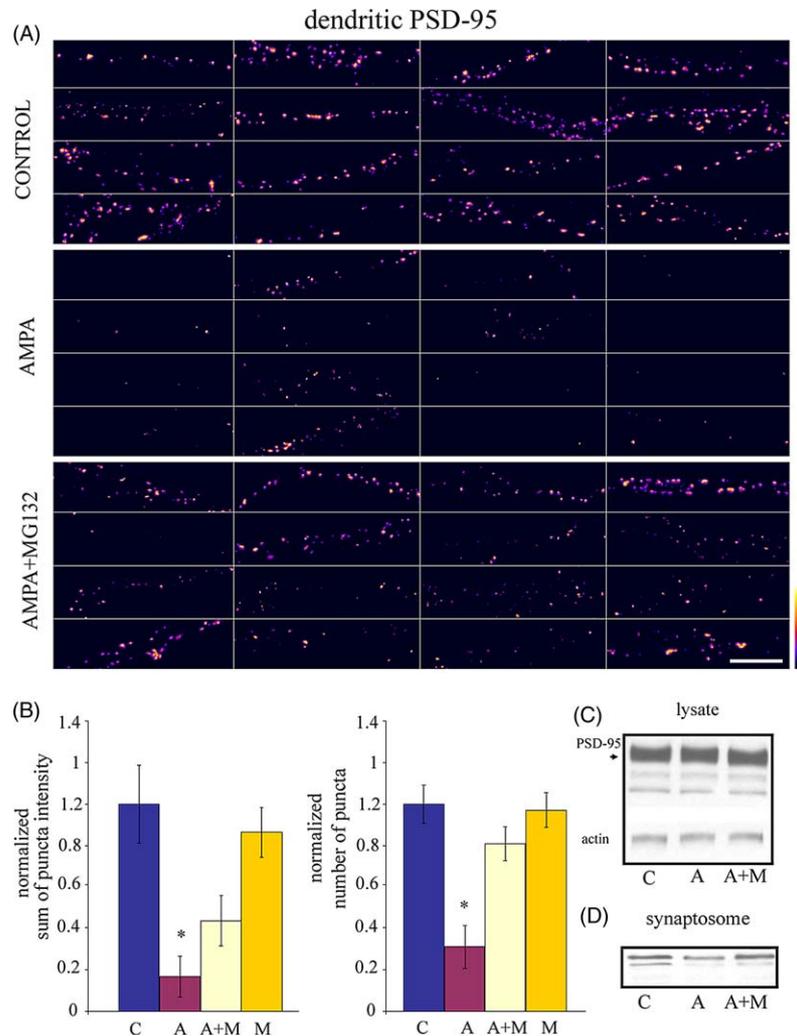


Fig. 2. PSD-95 exhibits a proteasome-sensitive downregulation in protein level following AMPA stimulation. (A) PSD-95 immunocytochemistry in dendrites from vehicle treated (top panel), AMPA (middle panel) or AMPA and MG132 treated neurons (bottom panel). AMPA (100  $\mu$ M) stimulation was for 20 min and MG132 (50  $\mu$ M) was present 20 min before and during the entire period of AMPA stimulation. (B) Analysis of PSD-95 levels in control, AMPA (A) or AMPA + MG132 (A + M) conditions. AMPA stimulation decreases the number and total intensity of PSD-95 puncta in an MG132-sensitive manner. Each value is normalized to control ( $n = 16$  for the first three conditions and 20 for MG132 alone experiment,  $p < 0.05$ ). (C) Western blot analysis from hippocampal lysates stimulated with AMPA in the presence or absence of MG132 shows no apparent change in PSD-95 protein level. The arrow represents PSD-95 immunoreactivity. Lower blot represents the actin control. (D) Western blot analysis from hippocampal synaptosomes stimulated with AMPA shows a proteasome inhibitor-sensitive decrease in PSD-95 protein levels.

### 2.5. PSD-95 Western blotting and synaptosome stimulation

Two to three weeks old hippocampal cultures were lysed in RIPA buffer after incubation in MG132 (50  $\mu$ M, 20 min) before and during AMPA stimulation (100  $\mu$ M, 20 min). Equal amount of proteins from all conditions were run on a 4–15% gradient gel (Bio-Rad). After gel transfer, membranes were probed for PSD-95 and actin.

Synaptosomes were prepared from ~30 days old rat hippocampus as described in Bagni et al. (2000). Stimulation was performed in buffer containing 10 mM Tris, pH 7.5, 2.2 mM  $\text{CaCl}_2$ , 0.5 mM  $\text{Na}_2\text{HPO}_4$ , 0.4 mM  $\text{KH}_2\text{PO}_4$ , 4 mM  $\text{NaHCO}_3$ , and 80 mM NaCl.

Stimulation was performed at 37  $^\circ\text{C}$  with or without MG132 (50  $\mu$ M, 15 min) pre-treatment followed by AMPA treatment (100  $\mu$ M, 20 min) in the absence or presence of MG132.

### 2.6. Immunoprecipitation and GST-S5a chromatography

For immunoprecipitation and GST-S5a chromatography experiments, lysates from either two to three weeks old hippocampal cultures or whole hippocampus from Sprague–Dawley rats were used. GST-S5a affinity chromatography was performed as described earlier (Ehlers, 2003). Immunoprecipitations were performed as described in Colledge et al. (2003). Cultures were kept in MG132 (50  $\mu$ M, 20 min) before stimulation.

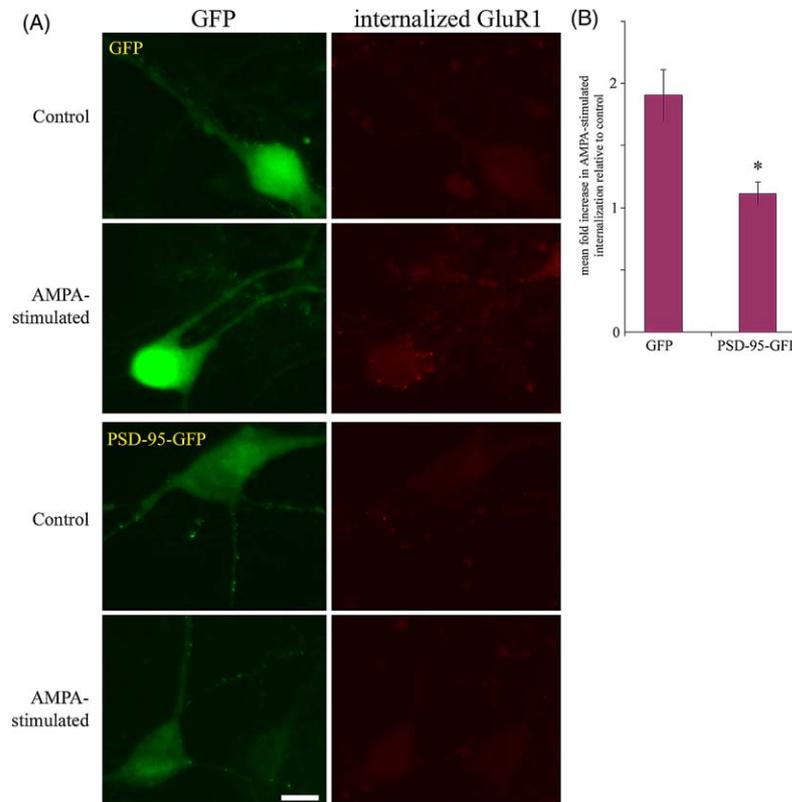


Fig. 3. PSD-95 overexpression reduces AMPA-induced GluR1 endocytosis. (A) Shown are the GFP signal (green) and internalized GluR1 signal (red) neurons expressing GFP alone (top four images) or PSD-95-GFP (bottom four images). In GFP-alone-expressing neurons AMPA stimulation lead to GluR1 internalization, whereas in PSD-95-expressing neurons AMPA stimulation did not result in GluR1 internalization. Scale bar = 5  $\mu$ m. (B) Analysis showing the AMPA-stimulated internalization of GluR1 in GFP-alone-expressing neurons and the lack of AMPA-stimulated internalization in PSD-95-GFP-expressing neurons ( $n = 30$  for each condition,  $p < 0.05$ ).

Stimulation was performed either with NMDA (20  $\mu$ M, 3 min followed by 10 min incubation (Fig. 4A–D) or for the “time course experiment” (Fig. 4E and F), 3 min stimulation followed by 0, 2, 7, 12 min incubation in conditioned media (Colledge et al., 2003) or AMPA (100  $\mu$ M, 20 min). For both GST-S5a chromatography and immunoprecipitations, lysis buffer contained 50  $\mu$ M MG132 and 1  $\mu$ M ubiquitin aldehyde (Calbiochem). Deubiquitinating enzyme application has been performed by applying 5  $\mu$ g each of isopeptidase-T (Calbiochem) and UCH-L3 (Affiniti Research, Exeter, UK) to the beads for 15 min at room temperature.

### 3. Results

A brief application of AMPA to cultured hippocampal neurons has been previously shown to induce a robust endocytosis of GluRs from the surface (Carroll et al., 1999; Lissin et al., 1999; Beattie et al., 2000; Lin et al., 2000; Zhou et al., 2001; Patrick et al., 2003). In order to investigate if PSD-95 levels are coordinately regulated with GluR endocytosis in dissociated hippocampal cultures, we induced GluR internalization with

AMPA stimulation and then examined both the internalized pool of GluR1 and PSD-95 using immunocytochemical techniques. If one examines many neurons in the same dish, it is clear that cells exhibit variable levels of internalized GluRs in response to AMPA stimulation ( $n = 4, 3,$  and  $4$  cells/dendrites that shows high ( $194.0 \pm 8.9$ ), medium ( $88.6 \pm 4.5$ ) and low ( $22.6 \pm 6.2$ ) level of internalized receptor puncta intensity, respectively) (e.g. Fig. 1B). We examined the relationship between the magnitude of internalized GluR and the degree of PSD-95 expression in individual dendrites. We found that cells that robustly internalized GluRs in response to AMPA treatment had significantly reduced levels of PSD-95 puncta (Fig. 1A, arrowheads). In contrast, the cells that did not respond to AMPA stimulation by internalizing receptor possessed noticeably higher levels of PSD-95 (Fig. 1A, arrows). Indeed, if one examines individual neurons that represent the full range of internalized GluR (no internalized puncta to high levels of internalized puncta) one consistently observes an inverse correlation with PSD levels (Fig. 1B,C). These data suggest a link between the absence of PSD-95 and the presence of internalized GluR1. These data are consistent with the idea that

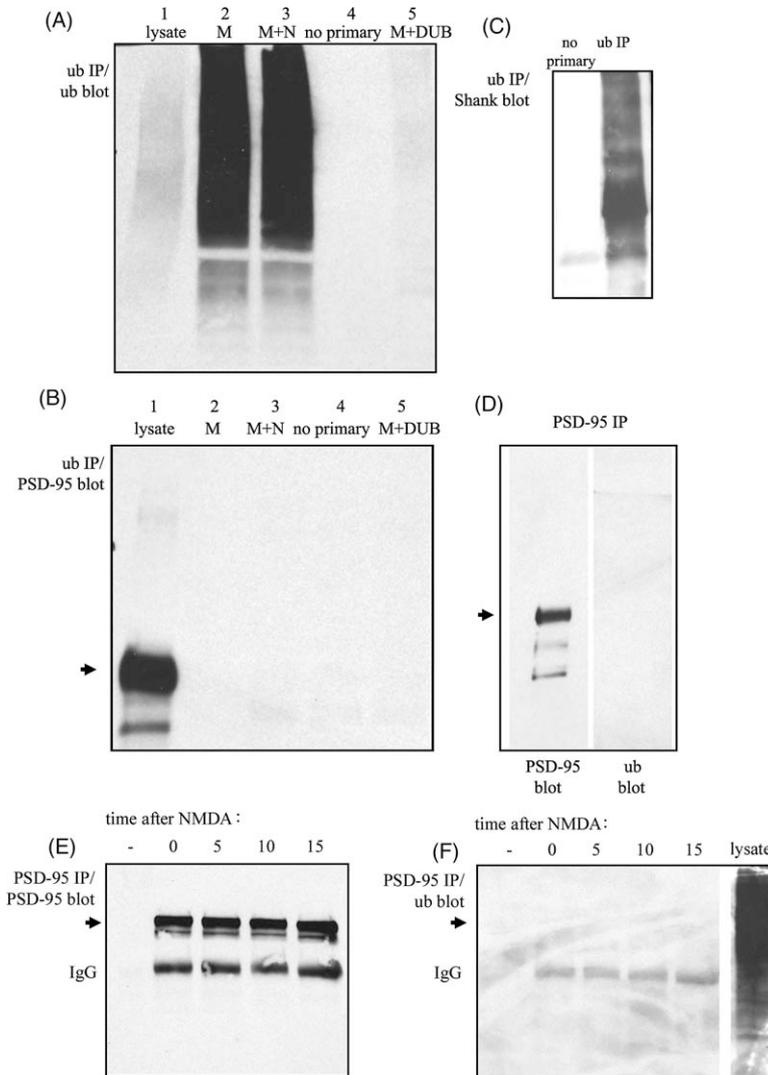


Fig. 4. The ubiquitination of PSD-95 cannot be detected. A series of experiments using immunoprecipitation followed by Western blot analysis failed to reveal the ubiquitination of PSD-95. (A) Lysates prepared from cultured hippocampal neurons were subject to immunoprecipitation with an anti-ubiquitin antibody and then probed with anti-ubiquitin antibody. Treatment with MG132 (lane 2) or MG132 + NMDA (lane 3) increased the abundance of ubiquitinated proteins relative to untreated lysates. Treatment of the beads with a deubiquitination enzyme abolishes the ubiquitin signal (lane 5). (B) The same blot as shown in A, reprobed with rabbit polyclonal PSD-95 antibody. Although PSD-95 could be detected in the lysate (lane 1), no polyubiquitination of PSD-95 was observed in any of the treatment conditions (lanes 2–5). (C) A blot from ubiquitin IP showing that Shank is ubiquitinated. (D) A reverse immunoprecipitation experiment in which PSD-95 is immunoprecipitated from lysates treated with MG132 + NMDA and then Western blot analysis is conducted with either an anti-PSD-95 antibody (left lane) or an anti-ubiquitin antibody. The success of the immunoprecipitation is shown by the presence of PSD-95 in the PSD-95 blot; however, no PSD-95 is detected in the anti-ubiquitin blot. (E) Time course experiment in which the PSD-95 pull-down was conducted at variable intervals (min) following the initiation of NMDA stimulation (3 min total NMDA stimulation). The arrow represents PSD-95 immunoreactivity. IgG is also indicated. (F) The same blot shown in E reprobed using an anti-ubiquitin antibody. No ubiquitinated PSD-95 was detected at any of the intervals examined, although an abundance of ubiquitinated proteins is detected in the lysate (not subjected to PSD-95 IP). The arrow indicates the size of PSD-95 on the gel.

AMPA stimulation leads to a loss of PSD-95 and the internalization of GluR1. It is attractive to propose that AMPA stimulation leads to a degradation of PSD-95 that precedes the internalization of the glutamate receptor. However, the inverse correlation between levels of internalized GluR and PSD-95 (Fig. 1C) could also be explained by pre-existing differences in the levels of PSD-95. Suppose, for example, that a subpopulation of neurons begin with less

PSD-95 and less surface GluR available for endocytosis. Alternatively, PSD-95 might exert a stabilizing influence on GluRs such that low levels of PSD-95 predispose receptors to internalize.

In order to test for the first possibility, we examined the amount of surface GluR1 present in neurons that have high, medium or low levels of PSD-95 in the absence of stimulation. We did not find a correlation between PSD-95 level and surface GluR1 level

(Fig. 1D). This observation indicates that the reduced levels of PSD-95 observed in neurons with internalized GluR1 (Fig. 1A) is not due to a pre-existing relationship between PSD-95 levels and surface receptors. Furthermore, Schnell et al. (2002) have shown that in organotypic slice cultures, PSD-95-overexpressing cells do not show a change in the response to bath-applied AMPA compared to non-transfected cells. These data thus leave open the possibility that PSD-95 levels are decreased upon AMPA treatment and that this decrease is important for subsequent GluR internalization.

Recent work has shown that the ubiquitin–proteasome pathway plays a major role in regulating synaptic function (Hedge et al., 1997; Jiang et al., 1998; DiAntonio et al., 2001; Ehlers, 2003; Pak and Sheng, 2003; Patrick et al., 2003; Speese et al., 2003; Zhao et al., 2003; Colledge et al., 2003; Liu et al., 2004; McCabe et al., 2004). In order to test if the AMPA-stimulated PSD-95 decrease observed above is regulated by ubiquitin–proteasome pathway, we have treated cultured hippocampal neurons with AMPA in the presence and absence of proteasome inhibitor MG132 (Lee and Goldberg, 1998) and monitored PSD-95 levels immunohistochemically. AMPA stimulation caused a significant decrease in both the number and the total intensity of PSD-95 puncta in hippocampal dendrites (Fig. 2A,B). The decrease in PSD-95 level was sensitive to a proteasome inhibitor suggesting that the ubiquitin–proteasome pathway—directly or indirectly—participates in this AMPA-induced decrease in PSD-95. In addition, we conducted Western blot analysis from either hippocampal lysates or synaptosomes treated with AMPA. Although an AMPA-stimulated decrease in PSD-95 was not observed in the whole hippocampal lysates (Fig. 2C), a proteasome-inhibitor-sensitive decrease in PSD-95 levels was detected in synaptosomes (Fig. 2D).

If PSD-95 is regulated by ubiquitin–proteasome pathway, overexpression of PSD-95 might saturate the enzymatic machinery required to downregulate PSD-95. Furthermore, if PSD-95 downregulation has a role in GluR1 endocytosis, then overexpression of PSD-95 should also affect GluR endocytosis. In order to investigate these possibilities, we expressed PSD-95-GFP or GFP alone in cultured hippocampal neurons and examined GluR-endocytosis in response to AMPA stimulation. Neurons that expressed GFP alone showed a significant increase in internalized GluR1 following stimulation with AMPA (Fig. 3A,B). In contrast, neurons that expressed PSD-95-GFP did not exhibit significant AMPA-stimulated endocytosis of GluR1. Although we cannot rule out the possibility that PSD-95 overexpression results in increased recycling, these results suggest that a limited concentration of PSD-95 is essential for neurons to exhibit AMPA-stimulated GluR1 endocytosis.

The ubiquitin–proteasome system marks the target proteins to be degraded by the attachment of a polyubiquitin chain. We have previously shown that polyubiquitination is required for GluR endocytosis (Patrick et al., 2003) and hypothesized that the target for the proteasome is a protein that interacts directly or indirectly with the GluRs. As such, the target protein should exhibit AMPA-stimulated polyubiquitination. One common way to determine if a protein is ubiquitinated is to pull-down ubiquitinated proteins and then conduct Western blot analysis for the protein of interest (e.g. Ehlers, 2003; Colledge et al., 2003). We have tried two methods to detect ubiquitinated proteins. The first involves immunoprecipitation (with antibodies to ubiquitin or candidate proteins) and the second is GST-S5a affinity chromatography (Layfield et al., 2001). S5a is a subunit of proteasome that has been shown to bind to polyubiquitin chains (Deveraux et al., 1994). Using either cultured hippocampal neuron lysates (Fig. 4) or whole hippocampal lysates (data not shown) we found that we could isolate an abundance of ubiquitinated proteins by either ubiquitin immunoprecipitation followed by Western blot analysis using an anti-ubiquitin antibody (Fig. 4A) or GST-S5a chromatography (data not shown). The amount of ubiquitinated proteins that we could isolate was enhanced by a brief (20 min) treatment with MG132 (50  $\mu$ m) or MG132 + NMDA stimulation. We addressed the specificity of our assay by treating the pull-down beads with a deubiquitinating enzyme (DUB) that removes the ubiquitin chains from ubiquitinated proteins. DUB application to the beads from either immunoprecipitation removed all of the polyubiquitin signal (Fig. 4A).

Although we have shown that we were able to pull-down ubiquitinated proteins (Fig. 4A), we were unable to observe the ubiquitination of PSD-95 when the same blots were reprobed with an anti-PSD-95 antibody (rabbit polyclonal; Fig. 4B). Similar experiments conducted with two additional PSD-95 antibodies also yielded negative results. We were, however, able to detect the polyubiquitination of Shank (Ehlers, 2003) using an anti-Shank antibody after a ubiquitin immunoprecipitation (Fig. 4C). We also attempted to demonstrate the ubiquitination of PSD-95 by conducting a reverse immunoprecipitation experiment, in which we first immunoprecipitate PSD-95 using an anti-PSD-95 antibody and then probe the blot with either a PSD-95 or ubiquitin antibody (Fig. 4D) (e.g. Colledge et al., 2003). Although we could clearly detect the presence of PSD-95 with the anti-PSD-95 antibody, we did not see any ubiquitin immunoreactivity when the anti-ubiquitin antibody was used. Also, an additional set of immunoprecipitations with different PSD-95 antibodies yielded same negative results. These results are consistent with one study in which GST-S5a did not pull-down PSD-95 from cortical culture lysates (Ehlers,

2003). Colledge et al. (2003), however, reported the ubiquitination of PSD-95 from stimulated cultures. They stimulated hippocampal cultures with NMDA (20  $\mu$ M for 3 min) and observed the polyubiquitination 10 min following NMDA stimulation. We used the same techniques and antibodies as Colledge et al. (2003) (Fig. 4D), but did not observe the ubiquitination of PSD-95 from AMPA- (data not shown) or NMDA-stimulated cultures, including experiments in which we looked for ubiquitination at different intervals following NMDA stimulation (Fig. 4E). In addition, we considered the possibility that the activity of deubiquitinating enzymes during our assay might remove polyubiquitin chains; to address this we routinely included DUB inhibitors during pull-downs to increase the pool of ubiquitinated PSD-95 species. Unfortunately, we were still not able to detect ubiquitination of PSD-95. These results suggest that either the ubiquitination of PSD-95 is extremely transient or that PSD-95 may not be a direct target of UPS, instead PSD-95 levels may be regulated by other bona fide and direct proteasomal targets.

#### 4. Discussion

In this paper, we show that there is a proteasome-dependent downregulation of PSD-95 levels in response to AMPA stimulation. This decrease was observed with both immunocytochemical techniques labeling PSD-95 clusters in dendrites and a Western blot analysis of PSD-95 from stimulated synaptosomes. We observed a negative correlation between AMPA-stimulated internalized GluR1 and PSD-95 staining. We also demonstrated a proteasome-dependent AMPA-stimulated decrease in the number and intensity of the PSD-95 puncta. By itself, the observed inverse correlation between PSD-95 and AMPAR does not indicate whether PSD-95 degradation is up or downstream of GluR internalization. The blockade of GluR internalization by PSD-95 overexpression, however, suggests that degradation of PSD-95 is upstream of internalization.

These data point to the possibility that PSD-95 may be a target of UPS. If PSD-95 is a direct target of UPS, then it should be polyubiquitinated. Using standard techniques to detect the polyubiquitination of a protein, we were unable to observe a polyubiquitinated PSD-95 species from lysates prepared from either cultured hippocampal neurons or hippocampal slices. It is possible that PSD-95 requires activity to be ubiquitinated as shown by Colledge et al. (2003), but we were not able to detect polyubiquitinated PSD-95 from cultures stimulated with NMDA (the same protocols that Colledge et al. used) or AMPA. It is possible that our failure to observe activity-stimulated PSD-95 ubiquitination reflects differences in culture conditions, or the

transient nature of PSD-95 ubiquitination. If the ubiquitination of PSD-95 is transient, however, treatment with a proteasome inhibitor might be expected to stabilize the ubiquitinated population. This was not observed in our experiments. Taken together our data suggest that PSD-95 may be an indirect target of UPS regulation. For example, Ehlers (2003) observed other PSD proteins that are not ubiquitinated but whose levels can be regulated by activity in a proteasome-dependent manner. Identifying the bona fide targets of the proteasome and the mechanisms by which these targets regulate proteins like PSD-95 will be the focus of future experiments.

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