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AMPA Receptor Trafficking and Synaptic Plasticity Require SQSTM1/p62

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Abstract

SQSTM1/p62 is a multidomain/scaffold for the atypical protein kinase Cs (aPKC). Phosphorylation of AMPA receptors by PKC has been shown to regulate their insertion in the postsynaptic membrane. Here, we directly tested whether p62 could interact with AMPA receptor subunits and influence their trafficking and phosphorylation. GluR1 receptor intracellular loop L2–3 and the ZZ-type zinc finger domain of p62 are essential for the interaction between these two proteins. In this context, both p62 and aPKC-mediated phosphorylation were necessary for surface delivery of the receptor. Our findings reveal that p62 is the first protein identified that interacts with a region of the GluR receptor other than the C-terminal tail. Furthermore, mice deficient in p62 displayed impaired hippocampal CA1 long-term potentiation (LTP), along with diminished surface expression of GluR1 and phosphorylation of S818. Lastly, we identify a conserved sequence (ISExSL) shared by all p62 interacting-aPKC substrates. These findings support a model where p62 interaction and aPKC phosphorylation act together to mediate AMPA receptor trafficking and long-term synaptic plasticity in the hippocampus. © 2008 Wiley-Liss, Inc.

Keywords

GluR1; aPKC; LTP; phosphorylation; surface delivery

INTRODUCTION

α -amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA)-type glutamate receptors mediate the fastest excitatory synaptic transmission in the mammalian brain. Structurally, AMPA receptors are heterotetrameric cation channels composed of four subunits: GluR1–4, each of which comprises about 900 amino acids with a molecular weight of about 105 kDa, and shares 68–74% amino acid sequence identity. Each AMPA receptor subunit consists of an extracellular N-terminus, three transmembrane spanning domains (TM1, TM3 and TM4), a re-entrant transmembrane domain (TM2), and an intracellular C-terminus. Therefore, the transmembrane domains form two intracellular loops (L1–2 and L2–3) and one extracellular

loop (L3–4). The re-entrant TM2 domain contributes to the cation pore channel. The C-terminal cytoplasmic tail of each AMPA receptor subunit is unique and could be associated with specific regulatory proteins, which are related to receptor modification, trafficking, and signaling (Hollmann and Heinemann, 1994; Dingledine et al., 1999).

It is widely believed that long-term potentiation (LTP) and long-term depression (LTD), form the cellular and molecular basis for neuronal plasticity, including learning and memory (Bliss and Collingridge, 1993; Malenka and Nicoll, 1999; Malinow and Malenka, 2002). Trafficking of AMPA receptors to and away from synapses is a mechanism to modulate synaptic strength (Lynch and Baudry, 1984; Malenka and Nicoll, 1999). During LTP expression, additional AMPA receptors are delivered to the postsynaptic membrane (Shi et al., 1999). In contrast, LTD induces receptor internalization (Sheng and Lee, 2001). Therefore, alterations in synaptic strength are directly related to the receptor exocytosis and endocytosis (Malinow and Malenka, 2002). AMPA receptor trafficking to the surface is primarily regulated by two mechanisms: (1) receptor associated proteins that aid in delivery of the receptor to the surface membrane (Hayashi et al., 2000; Fukata, 2005; Palmer et al., 2005), and (2) phosphorylation (Roche et al., 1996; Song and Haganir, 2002; Lee et al., 2000, 2003; Oh et al., 2006; Shepherd and Haganir, 2007). For example, two phosphorylation sites in the C-terminus of GluR1 subunit exist, S845 is a protein kinase A (PKA) site, whereas S831 is a site phosphorylated by calcium/calmodulin-dependent protein kinase II (CaMKII) and protein kinase C (PKC). Recently, an additional phosphorylation site at S818 has been identified for PKC (Boehm et al., 2006). However, the detailed cellular and molecular mechanism for AMPA receptor trafficking is not yet fully understood.

Several members of the PKC family directly phosphorylate AMPA receptor subunits to mediate plasticity (Carvalho et al., 1999; Chung et al., 2000; McDonald et al., 2001; Correia et al., 2003; Boehm et al., 2006; Lee et al., 2007). The hippocampus is the primary brain region responsible for learning and memory. The atypical PKC (aPKC) PKC δ and PKM ζ are two abundant atypical PKC isoforms expressed in hippocampus (Hernandez et al., 2003; Oster et al., 2004). Likewise, the aPKC scaffold, p62/Sequestosome 1 (SQSTM1), is also primarily expressed in hippocampus (Gong et al., 1999). However, p62 binds to the regulatory region of the aPKCs (Puls et al., 1997; Sanchez et al., 1998), whereas PKM ζ lacks this region and therefore would not be expected to interact with p62. Furthermore, mice deficient in p62 exhibit loss of numerous hippocampus-dependent behaviors (Babu et al., 2008). The possibility that the aPKC adaptor functions as a scaffold for aPKC-mediated phosphorylation of the AMPA receptor and possible regulation of trafficking has not been investigated. p62 is a cytoplasmic and membrane-associated protein that possesses six domains: a PB1 domain consisting of SH2 binding motif and aPKC interacting domain (AID), a ZZ-type Zinc finger domain for interaction with aPKC substrates, a tumor necrosis factor receptor-associated factor 6 binding domain, two PEST domains for proteolytic recognition, and a ubiquitin-associated (UBA) domain (Moscat et al., 2007). These domains endow p62 with the ability to associate with many other proteins and enables p62 to serve as a scaffold to recruit substrates of aPKC through its PB1 domain and ZZ-type finger domain (Puls et al., 1997; Gong et al., 1999; Sanz et al., 1999; Cariou et al., 2002; Croci et al., 2003; Kim, 2006). In addition, p62 has also been shown to function as a shuttling protein for endocytosis of polyubiquitinated proteins through interaction with its UBA domain (Geetha et al., 2005).

Here we demonstrate that p62 is an AMPA receptor interacting protein (RIP). The interaction between p62 and AMPA receptor is mediated through the AMPA receptor subunit intracellular loop L2–3 and the ZZ-type Zinc finger domain of p62. Furthermore, LTP was significantly reduced in mice lacking p62 along with a parallel decrease in surface GluR1 and GluR1-pS818 phosphorylation. Altogether, our results reveal that p62 and aPKC play a critical role in synaptic plasticity via regulating AMPA receptor trafficking and phosphorylation.

MATERIALS AND METHODS

Generation of p62 Knock-Out Mice

Knock out (KO) mice (p62^{-/-}) were generated as described previously (Duran et al., 2004). For the duration of the study, all mice were housed in a pathogen free barrier environment. The animals were handled according to the NIH and Auburn University IACUC guidelines.

Antibodies

The mouse GluR1 and GFP monoclonal, rabbit HA, and Myc polyclonal antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). GluR1 polyclonal, GluR2 and GluR3 monoclonal antibodies, GluR1 phosphorylation antibody pS818 were a kind gift from R.L. Huganir from Johns Hopkins University (Baltimore, MD). P62 monoclonal antibody and PKC λ monoclonal antibody were from BD Biosciences (San Jose, CA). Rabbit p62 polyclonal antibody was raised against the full-length p62 (Geetha et al., 2005).

Generation of GluR1 Deletion Constructs

The GluR1 cDNA deletions were performed with a Quick-Change® II XL Site-Directed Mutagenesis Kit according to the manufacturer's standard protocol (Stratagene, La Jolla, CA). The primers used for loop L1–2 deletion: Forward 5'-GGAGT GAGCGTCGTCCTCTTCCTGGT CAGCTTTGGCATATT CAACAGCCTGTGGTTCTCC-3'; Backward 5'-GGAGAAC CACAGG CTGTTGAATATGCCAAAGCTGACCAGGAA GAGGACGACGCTCACTCC-3'. The primers used for loop L2–3 deletion: Forward 5' -CCCTGGGGGCCTTCATGCAG CAAGGATGTATCG TCGGCGGCGTCTGGTGGTTCTTC AC-3'; Backward 5'-GTGAAGAACCACCAGACGCCGC CG ACGATACATCCTTGCTGCATGAAGGCCCCAGGG-3'. The deletions were confirmed by DNA sequencing and the absence of any nonspecific mutation was confirmed for the GluR1 cDNA.

Preparation of Mouse Brain Lysate

Adult mouse brains were homogenized in ice-cold Triton lysis buffer [50 mM Tris-HCl pH 7.5, 150 mM NaCl, 10 mM NaF, 0.5% Triton X-100, 1 mM Na₃VO₄, 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 μ g/ml leupeptin, and 2 μ g/ml aprotinin] plus SDS at different concentration range. The homogenate was sonicated on ice for 10 s three times, followed by rotating the lysates for 30 min, and centrifuged for 10 min at 48°C. The protein concentration was estimated by Bio-Rad DC assay (Hercules, CA), with 1 mg/ml bovine serum albumin (Sigma-Aldrich, St. Louis, MO) as a standard.

HEK Cell Culture and Transfection

Human embryonic kidney (HEK) 293 cells were maintained as previously described (Geetha et al., 2005). The HEK cells were transfected with the calcium phosphate method by using a Mammalian Cell Transfection Kit (Millipore, Billerica, MA) or with cationic lipid method by using Lipofectamine™ 2000 Transfection Reagent (Invitrogen, Carlsbad, CA). The cells were harvested and lysed with Triton lysis buffer (Geetha et al., 2005). Protein concentration was estimated by Bradford assay.

Coimmunoprecipitation and Western Blotting

HEK Cell lysates (750 μ g) or brain lysates (1 mg) were diluted in 1 ml lysis buffer and incubated with 4 μ g of primary antibodies at 4°C for 3 h. The immunoprecipitates were collected with agarose-coupled secondary antibodies (Sigma) for at least 2 h and then were washed three times with lysis buffer. Proteins bound to the antibodies were released with protein sodium

dodecyl-sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer (2% SDS, 1% β -mercaptoethanol, 0.005% Bromophenol blue, 2% glycerol, and 0.05 M Tris-HCl, pH 6.8). The brain lysates, cell lysates, brain-slice lysate, or immunoprecipitated proteins were resolved by 10% SDS-PAGE under reducing conditions (1% β -mercaptoethanol) and transferred onto nitrocellulose membranes (Amersham Biosciences). The blots were incubated with appropriate antibodies at 4°C overnight, followed by incubation with horseradish peroxidase-conjugated secondary antibodies (Amersham Biosciences) the blots were developed by enhanced chemiluminescence and Hyperfilm (Amersham Biosciences). The captured images were scanned and quantified using the UN-SCAN-IT gel software (Silk Scientific, Orem, UT).

Immunocytochemistry

For localization of AMPA receptor subunits and p62, HEK cells were cotransfected with Myc- or HA-tagged p62 and GluR1, GluR2, or GluR3 with or without GFP tag as indicated in the figures. After 48 h, the cells were fixed, permeabilized, and incubated with rabbit anti-Myc or HA IgG and mouse anti-GluR1, GluR2, or GluR3 IgG for the AMPA receptor subunit construct without GFP tag. The cells were labeled with Texas Red-conjugated antirabbit antibodies (red) and Oregon Green-conjugated antimouse antibodies (Green). Localization was determined by confocal immunofluorescence microscopy and analyzed on a Bio-Rad MRC 1024 Laser Scanning Confocal Microscope.

Hippocampal Slice Preparation

Adult mice were anesthetized, decapitated, and brains were rapidly isolated in ice cold dissection buffer (250 mM sucrose, 25 mM NaHCO₃, 25 mM glucose, 2.5 mM KCl, 1.25 mM NaH₂PO₄, 2 mM CaCl₂, and 1.5 mM MgCl₂, pH 7.3). Hippocampal slices (transverse, 300 μ m thickness) were cut in ice cold dissection buffer continuously bubbled with a mix of 95% O₂ and 5% CO₂ using a Tissue Sectioning System (Vibratome, St. Louis, MO). The slices were then transferred to an incubating chamber filled with artificial cerebrospinal fluid (ACSF) (124 mM NaCl, 25 mM NaHCO₃, 25 mM glucose, 2.5 mM KCl, 1.25 mM NaH₂PO₄, 2 mM CaCl₂, and 1.5 mM MgCl₂, pH 7.3), equilibrated with a mix of 95% O₂ and 5% CO₂, and incubated at 22–24°C for 1.5 h before use.

Electrophysiology

Field excitatory postsynaptic potentials (fEPSPs) from the Shaffer collateral-CA1 synapses in hippocampal slices from adult mice were recorded at 22–24°C (Bukalo and Dityatev, 2006). Current intensity of test stimuli was set to produce 50% of subthreshold maximum and the test stimuli was applied every 15 s. Stimulus response and paired pulse ratio experiments were performed by varying the stimulus intensity and interpulse interval. LTP was induced by five trains of theta burst stimulation (TBS) (Each train consisted of 10 bursts at 5 Hz; each burst consisted of four pulses delivered at 100 Hz with 15 s interval). Values of LTP were calculated as the increase in the mean slopes of fEPSPs measured 50–60 min after TBS. NMDA receptor mediated fEPSPs were evoked in low Mg²⁺ (0.1 mM) ACSF supplemented with 10 mM 2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline (NBQX) purchased from Tocris, Ellisville, MO.

Surface Biotinylation

To measure surface level of GluR1 biotinylation was conducted essentially as described (Chan et al., 2006). In brief, acute hippocampal slices from adult mice were transferred to ice-cold ACSF for 2 min, followed by biotinylation in 1 mg/ml of EZ-Link Sulfo-NHS-SS-Biotin (Pierce) for 45 min with slow agitation at room temperature. The slices were rinsed in cold Tris-based ACSF (HEPES replaced by Tris) for three times to quench free biotin. The slices

then lysed in cold homogenized buffer (50 mM NaCl, 10 mM EDTA, 10 mM EGTA, 1 mM Na₃VO₄, 50 mM NaF, 25 mM NaPPi, 1 mM β-glycerophosphate, 1 mM PMSF, 1 μM microcystine, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 1% Triton X-100, and 50 mM HEPES, pH 7.5). The homogenates were sonicated on ice for 5 s three times and rotated at 4°C for 1 h. The lysates were centrifuged for 10 min to pellet the insoluble fraction. The protein concentration was examined with the Bradford dye (Bio-Rad). Samples (40 μg) were subjected to SDS-PAGE and Western blot to examine the total GluR1 in the whole lysates (both surface and intracellular fractions). For surface GluR1 analysis, 400 μg protein of each sample in 500 μl homogenization buffer was precipitated with 50 μl of 50% avidin-agarose beads (Pierce, Rockford, IL) for 3 h at 4°C. The beads were pelleted and rinsed three times with homogenization buffer, followed by boiling in 60 μl 1X SDS sample buffer. The samples (surface fraction and total lysates) were subjected to SDS-PAGE and Western blotting with appropriate antibodies.

Statistics

Data were expressed as the mean ± standard error of mean (SEM). Statistical analyses were performed using Excel (Microsoft, Redmond, WA). *P*-values <0.05 were judged statistically significant.

RESULTS

p62 Interacts with AMPA Receptor Subunits

All PKC isoforms, including αPKCs, phosphorylate the AMPA receptor subunit GluR1 both in vitro and in vivo (Boehm et al., 2006; Lee et al., 2007). Therefore, we hypothesized an interaction between the AMPA receptor and the αPKC adaptor, p62, might occur. To examine this possibility, HEK cells were cotransfected with AMPA receptor subunit GluR1, GluR2, or GluR3 and Myc-tagged p62 cDNA constructs followed by immunoprecipitation with appropriate antibodies (Fig. 1A–C). Western blot revealed that the AMPA receptor subunits GluR1–3, which are expressed in the adult mammalian hippocampus, interact with p62 in vitro (Fig. 1–C). As an alternative means to examine interaction, colocalization of GluR1–3 and p62 were also tested by cotransfection of HEK cells, followed by immunofluorescent microscopy. GluR1, GluR2, and GluR3 colocalized with p62 at the cell surface (Fig. 1D).

Next, the interaction between p62 and AMPA receptor subunits GluR1–3 in brain lysates prepared from adult wild-type (WT; ^{+/+}) and p62 knock-out (KO; ^{-/-}) mice was analyzed via coimmunoprecipitation. AMPA receptor subunits GluR1–3 interacted with p62 in the brain lysate from the WT mice; whereas the p62/AMPA receptor interaction was abolished in the p62 knock-out mice (KO) (Fig. 2A–C). As control, an absence of GluR antibody failed to pull-down p62 (not shown). These results validate that p62 interacts with all AMPA receptor subunits expressed in the adult mouse hippocampus both in vitro and in vivo and suggests a physiological role for this protein.

Mapping the Interaction of p62 with GluR1 Subunits

p62 possesses six domains, which enable the protein to recruit many different interactors (Moscat et al., 2007). To investigate which region of p62 is responsible for AMPA receptor interaction, C-terminal and N-terminal truncated p62 constructs were used to examine protein–protein interaction in transfected HEK cells by coimmunoprecipitation (Fig. 3A). Interestingly, the C-terminal truncated p62 (ΔUBA) still interacted with GluR1; whereas the N-terminal truncated p62 (ΔPB1, ΔZZ) lost the capacity to interact with GluR1 (Fig. 3B). These findings indicate that the N-terminus of p62 is essential for AMPA receptor interaction. There are three functional regions located on the p62 N-terminus: SH2 binding site, AID motif, and ZZ-type Zinc finger domain. To further investigate which domain in the N-terminus of p62 interacts

with the AMPA receptor, a series of p62 deletion constructs were employed to test the capacity for GluR1 to interact with p62 by cotransfection of HEK cells and coimmunoprecipitation (Table 1). Among these p62 deletion constructs, the deletion of ZZ domain totally abolished the p62/GluR1 interaction. We conclude that the ZZ-type Zinc finger domain of p62 mediates interaction with the AMPA receptor. To examine if these interactions possessed a physiological consequence, we next examined whether p62 regulated GluR1 localization in transfected HEK cells by immunocytochemistry (Fig. 4A). Wild-type p62 and p62 (Δ UBA) colocalized with GluR1 in the cell membrane; whereas p62 (Δ ZZ) failed to colocalize with GluR1. Interestingly, expression of GluR1 with the Δ ZZ construct resulted in intracellular accumulation of GluR1 (Fig. 4A). These results indicate that interaction of GluR1 with the ZZ-type Zinc finger domain of p62 may be necessary for AMPA receptor surface expression. To further validate this observation, HEK cells were cotransfected with GluR1 along with p62 (WT or Δ ZZ) in the presence or absence of active/inactivate GST-aPKC as shown (Fig. 4B). To measure the surface level of GluR1 biotinylation was conducted (Chan et al., 2006) followed by Western blot of the avidin-agarose beads with GFP to detect GluR1. Expression of the constructs was examined in the total cell lysates by blotting with antibodies to GFP, GST, Myc, HA, and Tubulin. The expression of GluR1 on the surface to total was normalized and graphed (Fig. 4C). Although p62 alone increased the expression of GluR1 at the cell surface, inclusion of active aPKC resulted in a significant increase in GluR1 at the cell surface. Expression of the catalytically inactivate aPKC construct, impaired this response. Likewise, absence of the GluR1 interaction site in p62 (Δ ZZ) along with expression of active aPKC resulted in diminished surface expression of GluR1. Altogether, these findings reveal that p62 and aPKC play a coordinate role in regulating GluR1 surface expression.

Intracellular Loop L2–3 of GluR1 is Critical for p62 Interaction

To date, most AMPA receptor associated proteins have been discovered to interact with the intracellular C-terminus of the receptor (Malinow and Malenka, 2002; Shepherd and Huganir, 2007). Therefore, we hypothesized that p62 might also interact with the AMPA receptor subunit C-terminus. To test this possibility, a series of C-terminal truncated GluR1 constructs were employed to map the interaction between the GluR1 and p62 in transfected HEK cells by coimmunoprecipitation (Shen et al., 2000). Surprisingly, all C-terminal truncated GluR1 constructs were observed to interact with p62. Total truncation of the GluR1 C-terminus did not impair interaction with p62 (Table 2).

The three transmembrane domains of AMPA receptor subunit form three intracellular regions: C-terminus, loop TM1-TM2 (L1–2) and loop TM2-TM3 (L2–3) (Hollmann and Heinemann, 1994). As GluR1 C-terminus is not responsible for the interaction with p62, these two intracellular loops might be the p62 interaction site. To test this possibility, two GluR1 deletion constructs were generated: GluR1 (Δ L1–2) and GluR1 (Δ L2–3), which lacks the intracellular loop L1–2 and loop L2–3, respectively (Fig. 5A). Compared with the wild-type GluR1, deletion of L1–2 had no effect on GluR1/p62 interaction; whereas deletion of L2–3 significantly reduced the GluR1/p62 interaction to 78% of control (N 5 4, $P < 0.005$) (Fig. 5B). A residual degree of interaction was observed between the Δ L2–3 mutant and p62. As aPKC phosphorylates the C-term tail of GluR1 (Boehm et al., 2006), it is possible that endogenous aPKC may still recruit p62 to the GluR1 receptor or another C-term interacting protein may also interact with p62 as well. These results suggested that the intracellular loop L2–3 of GluR1 is critical for GluR1/p62 interaction. Alignment of GluR family members reveals that the intercellular loop L2–3 is completely conserved among all AMPA receptor subunits GluR1–4 (Fig. 5C). This finding is consistent with the observation that p62 interacts with all AMPA receptor subunits (Fig. 1A–D and Fig. 2A–C). The functional role for this interaction was tested in transfected HEK cells by immunocytochemistry. Interestingly, in the absence of the L1–2 region, GluR1 still colocalizes with p62 at the cell surface; whereas the deletion of L2–3, the

p62 interaction site, impaired colocalization between GluR1/p62 at the cell membrane (Fig. 5D). Likewise, deletion of GluR Δ L2–3 but not GluR Δ L1–2 along with active aPKC and p62 resulted in diminished surface expression of biotinylated GluR1 (not shown). These results further suggest that interaction between p62 and the AMPA receptor may be necessary and functionally relevant.

Absence of p62 Impairs LTP

AMPA receptors mediate most excitatory neurotransmission in the central nervous system. We demonstrate that p62 interacts with AMPA receptors in vitro and in vivo and overexpression of p62 increased surface expression of the receptor. Therefore, it is possible that p62 might play an important role in synaptic plasticity. In addition, mice lacking p62 exhibit defective spatial learning, along with deficits in other hippocampus dependent tasks (Babu et al., 2008). The effect of p62 on basal synaptic transmission and synaptic plasticity in the Shaffer collateral-CA1 synapses of the hippocampus was examined. There were no significant differences in evoked fEPSPs across a range of stimulus intensities in slices obtained from mice lacking p62 compared with wild-type (WT) (Fig. 6A). Paired pulse facilitation (PPF) is a transient form of presynaptic-dependent short-term plasticity that is believed to be the result of enhanced probability of synaptic vesicle release (Zamanillo et al., 1999). PPF in the p62 knock-out mice was normal (Fig. 6B), consistent with the normal basal synaptic transmission. To further evaluate the properties of these synapses, hippocampal CA1 LTP induced by TBS was measured (Fig. 6C). Compared with WT mice, the LTP was abolished in the p62 KO mice; whereas post-tetanic potentiation in p62 KO mice remains normal. As there were no significant changes in stimulus-response curves or paired-pulse facilitation, the failure to induce LTP is not likely due to impaired basal synaptic transmission. As the form of LTP studied here is dependent on NMDAR-mediated induction (Huber et al., 1995; Morgan and Teyler, 2001), we studied the fEPSPs evoked first by TBS and found similar levels in both WT and KO (Fig. 6D), suggesting that there was no difference in NMDAR responses during the TBS priming. In addition, to test whether NMDAR component of fEPSP is altered or not, basal synaptic transmission mediated by NMDAR in the presence of low Mg²⁺ and AMPAR antagonist, NBQX, was examined. In this case, basal synaptic transmission in the KO mice was not significantly different from that of WT (Fig. 6E). Altogether, these findings indicate that p62 interaction with GluR1 has a physiological role.

p62 Regulates AMPA Receptor Phosphorylation

Phosphorylation of GluR1 regulates AMPA receptor trafficking to and from the postsynaptic membrane (Roche et al., 1996; Song and Huganir, 2002; Lee et al., 2000, 2003; Oh et al., 2006). A newly identified PKC phosphorylation site, S818, in GluR1 has been reported, which is necessary for AMPA receptor surface delivery and maintenance of GluR1 at the postsynaptic membrane (Boehm et al., 2006). Interestingly, GluR1 S818 is phosphorylated by all PKC isoforms, including the aPKCs. As hippocampal LTP in the p62 knock-out mice was impaired, and interaction with p62 was necessary for cell surface delivery of AMPA receptor in HEK cells, we hypothesized that an absence of p62 may also have an effect on both the amount of total GluR1 and phosphorylated GluR1 at the postsynaptic membrane. To investigate this possibility, we performed surface biotinylation and immunoblot analysis of acute hippocampal slices from 6-month-old wild-type (WT) and p62 knock-out (KO) mice. In the absence of p62, the surface level of GluR1 subunit as well as the degree of GluR1 S818 phosphorylation was significantly reduced; whereas the total amount of GluR1 subunit in the p62 knock-out mice was similar to that expressed in wild-type mice (Fig. 7A,B).

DISCUSSION

Several proteins, such as Stargazin, 4.1N, AP2, PI3-kinase, have been reported to regulate receptor trafficking and synaptic plasticity through interacting with AMPA receptors either directly or indirectly (Reviewed in Jiang et al., 2007; Shepherd and Huganir, 2007). In this study, we identified a novel AMPA RIP, p62/SQSTM1. The ZZ-type zinc finger domain of p62 and the intracellular loop L2–3 of AMPA receptor subunit are critical for these protein–protein interactions. Interestingly, p62 is the first protein that has been discovered to interact with the GluR receptor outside of its C-terminus with an impact on surface delivery and expression of LTP.

SQSTM1/p62 possesses six functional domains, which endow the protein with an ability to interact with many different molecules to exert multiple-functions. To date, most p62-interacting proteins have been observed to interact with its N-terminal ZZ-type zinc finger domain or the C-terminal UBA domain (Puls et al., 1997; Gong et al., 1999; Sanz et al., 1999; Cariou et al., 2002; Croci et al., 2003; Geetha et al., 2005; Kim, 2006). The UBA domain of p62 interacts with K63-polyubiquitinated membrane-bound proteins to initiate ubiquitin-dependent receptor endocytosis (Geetha et al., 2005), whereas the ZZ-type zinc finger domain interacts with substrates of aPKC (Puls et al., 1997; Gong et al., 1999; Sanz et al., 1999; Cariou et al., 2002; Croci et al., 2003; Kim, 2006). Therefore, p62 as a scaffold likely enables the kinase, aPKC, and the substrate, GluR1, to form a ternary complex. It is possible that the ZZ domain coordinates a correct folding of p62 to create an interaction surface. Thus far, several receptors and nonreceptor proteins have been discovered to interact with the ZZ-domain of p62. Those proteins include: dopamine D₂ receptor (DAR), GABA_C receptor subunit q1–3, growth factor receptor-bound protein 14 (Grb14), RIP, and potassium channel subunit Kvb2 (Table 3) (Puls et al., 1997; Gong et al., 1999; Sanz et al., 1999; Cariou et al., 2002; Croci et al., 2003; Kim, 2006). p62 interacts with the intracellular loop (TM3-TM4) of GABA_C receptor, ID (intermediate domain) of RIP and PIR (phosphorylated insulin receptor) domain of Grb14; whereas in our study, the intracellular loop L2–3 of AMPA receptor subunits was revealed to be critical for p62 interaction. Alignment of all p62-interacting sites in each protein reveals a potential conserved consensus sequence, ISE_xSL (where x is any amino acid) (Fig. 8A). We hypothesize this site might serve as a putative protein interaction motif to recruit the substrate for phosphorylation by aPKCs. In fact, interactions of p62 with Kvβ2, GABA_C receptor, RIP, and Grb14 are necessary for phosphorylation mediated by aPKC (Table 3).

Receptor phosphorylation by CaMKII and PKC play critical roles in AMPA receptor trafficking. Four phosphorylation sites have been discovered in the GluR1 C-terminus: S818 and T840 are PKC sites, S831 is both a PKC and CaMKII site, and S845 is phosphorylated by both PKA and cGKII (cGMP-dependent protein kinase II) (Roche et al., 1996; Esteban et al., 2003, Lee et al., 2003, 2007; Boehm et al., 2006; Serulle et al., 2007). In our study, surface delivery of GluR1 was not completely absent in hippocampus from mice deficient in p62. Therefore, other kinases/scaffold proteins may compensate for the deficit in p62. However, studies in HEK cells reveal that aPKC promotes surface expression of the receptor to a greater degree than GluR1 coexpressed with p62 alone. Altogether, these findings suggest that p62 and aPKC act together to mediate surface delivery of GluR1. These findings are similar to what has recently been reported for the PICK1 scaffold and phosphorylation by the classical PKCs in expression of mGluR7 surface expression (Suh et al., 2008). Our findings are in keeping with the reported role for phosphorylation in stabilizing the AMPA receptor in the synaptic membrane to mediate plasticity (Lee et al., 2000). The aPKC isoform, PKMζ, has a well-defined role in mediating late-phase LTP (Pastalkova et al., 2006), whereas, these findings reveal that PKCι/ζ which interact with p62, likely regulates the early phase of LTP. This idea would be consistent with the translocation of PKCι/ζ early after a tetanus that induces LTP (Sacktor et al., 1993). Therefore, as a scaffold p62 recruits GluR1 for phosphorylation by aPKC

and also participates in trafficking to the membrane. In this context, p62 has been shown to interact with MAP1B a component of the cytoskeleton (Pankiv et al., 2007), and MAP1B knock-out mice are likewise deficient in LTP (Zervas et al., 2005). Thus, p62 may be directly involved in trafficking of the phosphorylated receptor to the cell surface by interaction with the cytoskeleton as well as in regulation of GluR1 phosphorylation by aPKC.

Collectively the electrophysiology and behavioral phenotype of p62 knock-out reveal a notable similarity with those reported for the GluR1 knock-out mice (Zamanillo et al., 1999; Reisel et al., 2002). Additionally, the p62 knock-out mice display mature-onset obesity (Rodriguez et al., 2006), which might amplify the deficits in AMPA receptor surface delivery and synaptic plasticity through oxidative stress. Altogether, our study reveals a novel and critical role for p62 along with the aPKC isoforms in AMPA receptor trafficking and synaptic plasticity.

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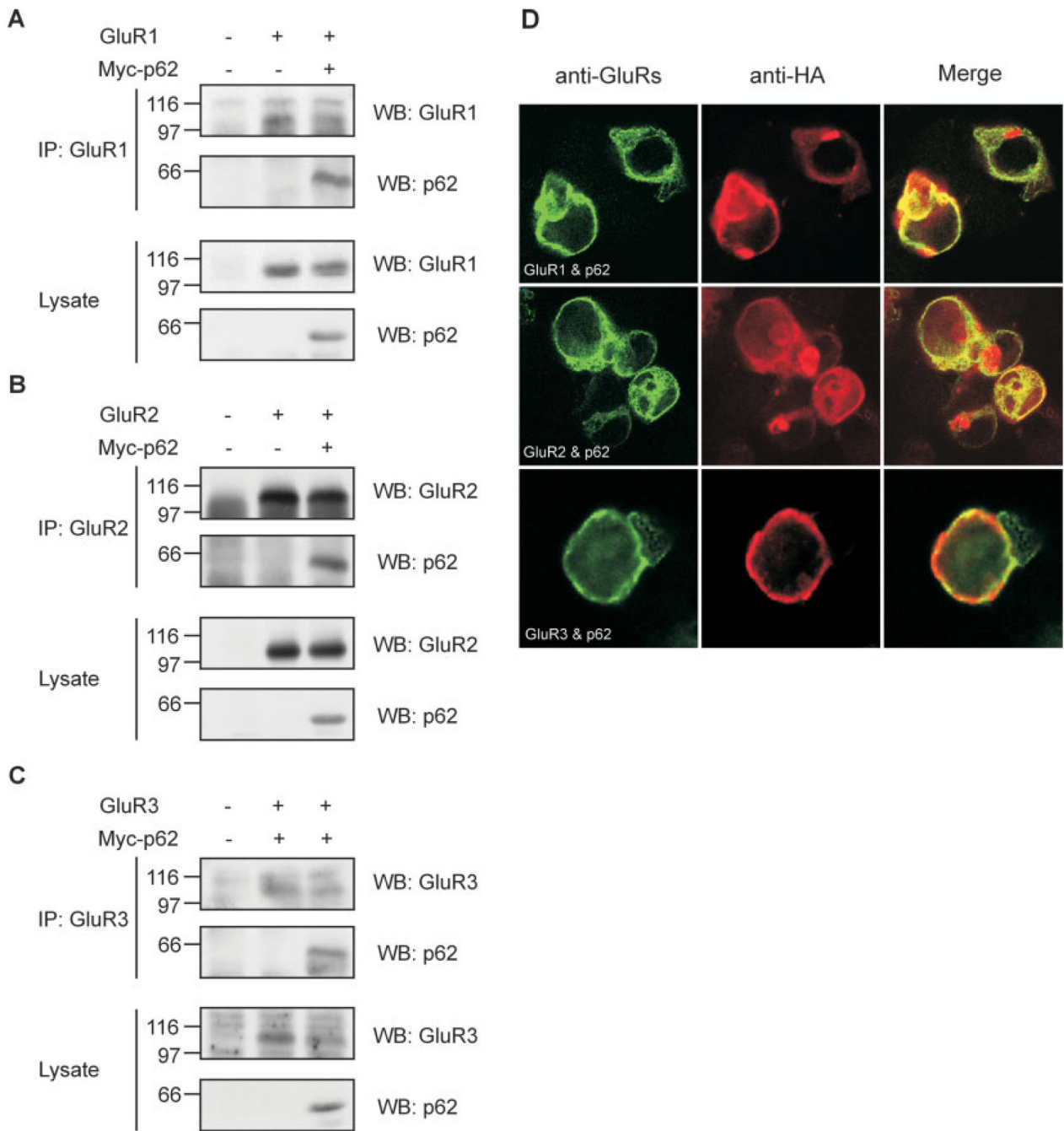
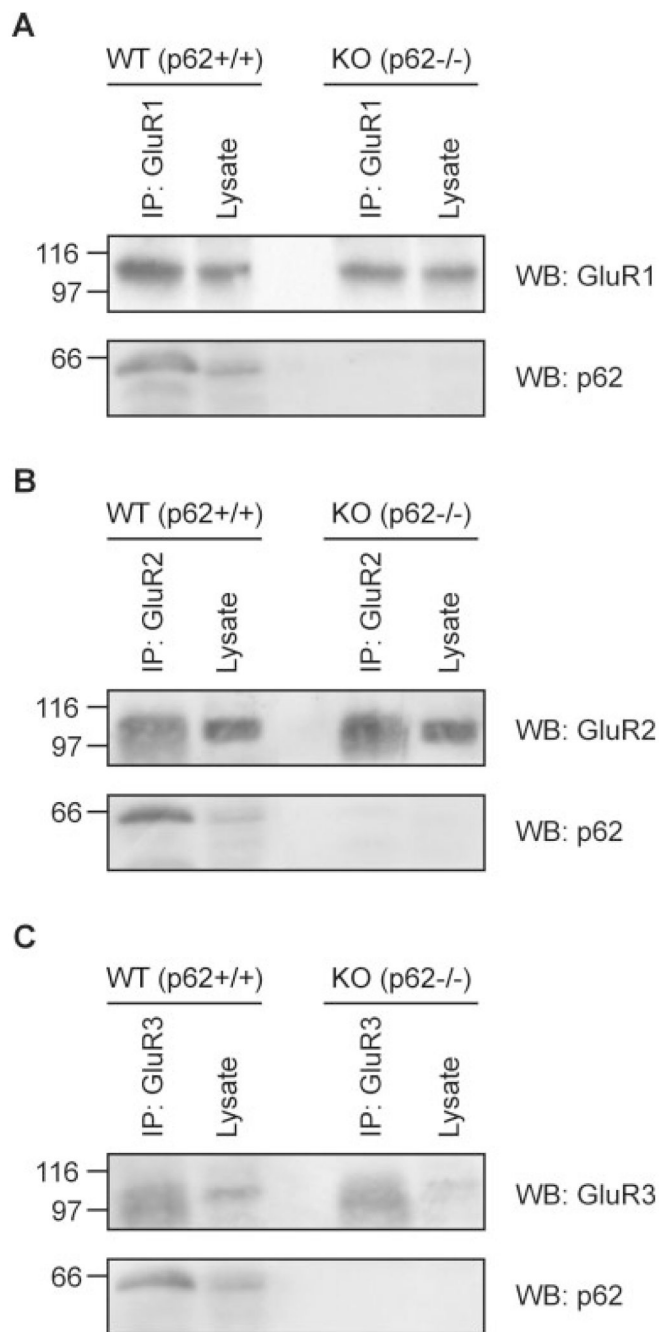


FIGURE 1.

p62 interacts with GluR1–3 subunits. A–C: HEK cells were cotransfected with GluR1, GluR2 or GluR3 and Myc-tagged p62. After 48 h, the cells were harvested and lysed with Triton lysis buffer. The cell lysates (750 µg) were immunoprecipitated with anti-GluR1, GluR2 or GluR3, and then Western blotted with anti-GluR1, GluR2 or GluR3, and anti-p62 (Upper panels). The lysates (50 µg) were also Western blotted with GluR1, GluR2 or GluR3, and p62 antibodies to verify expression of recombinant proteins (lower panels). D: HEK cells were cotransfected with GluR1 (upper panel), GluR2 (middle panel) or GluR3 (lower panel), and HA-tagged p62. After 48 h, the cells were fixed, and incubated with rabbit anti-HA IgG followed by labeling with Texas Red conjugated antirabbit antibodies (red) and mouse anti-GluR1, GluR2 or GluR3,

and labeled with Oregon Green conjugated antimouse antibodies (Green). The colocalization of GluR2 or GluR3 and p62 was determined by confocal microscopy. GluR1, GluR2, and GluR3 colocalize with p62 in the cell membrane (yellow). All experiments were repeated three independent times with similar results. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

**FIGURE 2.**

AMPA receptor subunits GluR1, GluR2, and GluR3 interact with p62. A–C: Brain lysates (1 mg) of wild-type (WT) and p62 knock-out (KO) were immunoprecipitated with anti-GluR1, anti-GluR2, and anti-GluR3, and then Western blotted with relevant AMPA receptor subunit antibodies, and anti-p62. The lysates (40 μ g) were also Western blotted with both AMPA receptor subunit and p62 antibodies. The findings are representative of three different experiments with similar results.

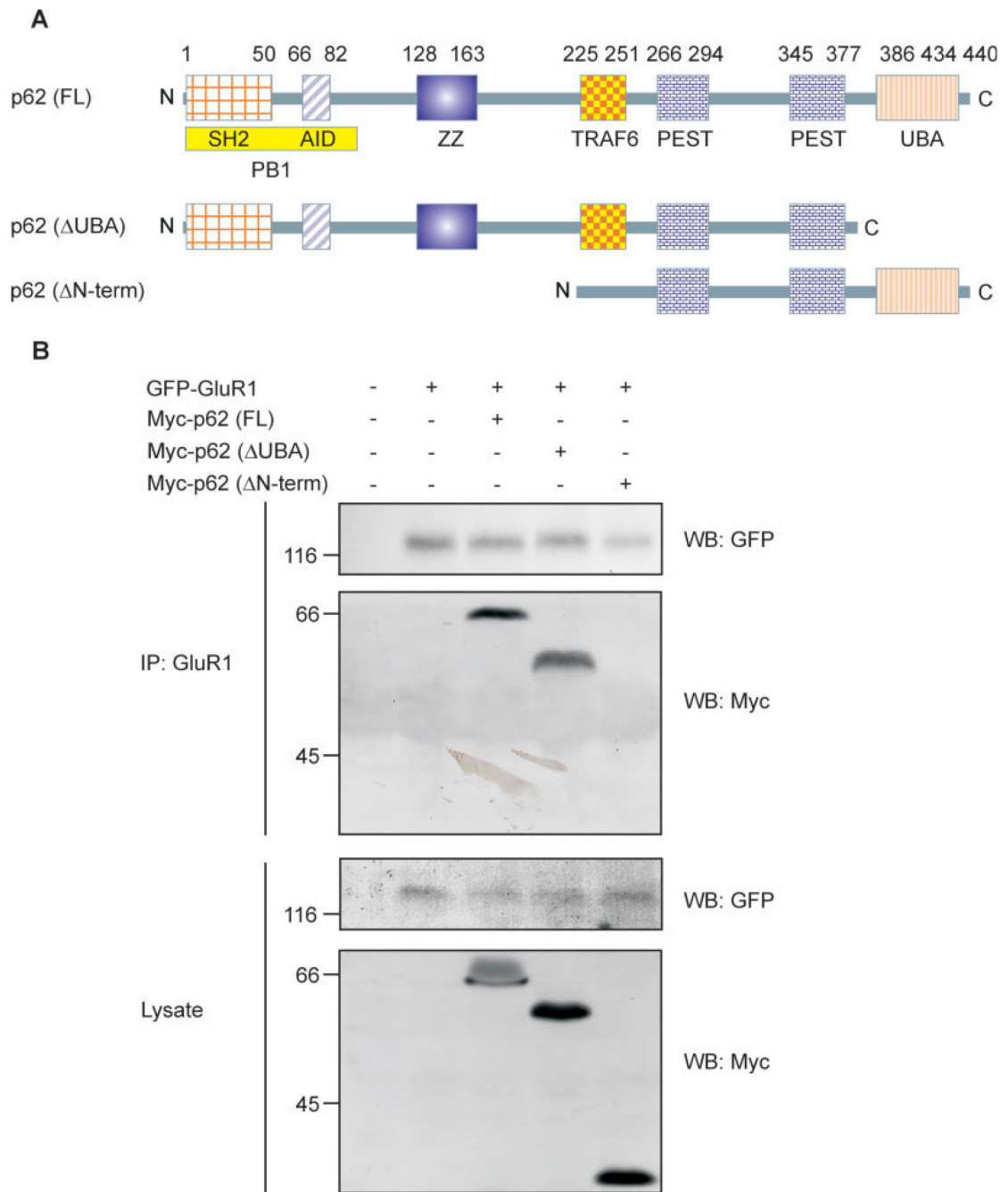


FIGURE 3.

N-terminus of p62 is responsible for AMPA receptor subunit interaction. A: Schematic diagram of Myc-tagged p62 constructs used for mapping of interaction between GluR1 and p62: p62 (WT), p62 (ΔUBA), and p62 (ΔN-term). B: HEK cells were cotransfected with GFP-GluR1 and Myc-tagged p62 (WT), p62 (ΔUBA), or p62 (ΔN-term). After 48 h, the cells were harvested and lysed with Triton lysis buffer. The cell lysates (750 μg) were immunoprecipitated with anti-GluR1, and then Western blotted with anti-GFP and anti-Myc (Upper 2 panels). The lysates (50 μg) were also Western blotted with anti-GFP and anti-Myc (Lower 2 panels) to verify expression of the constructs. These findings are representative of three similar

experiments. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

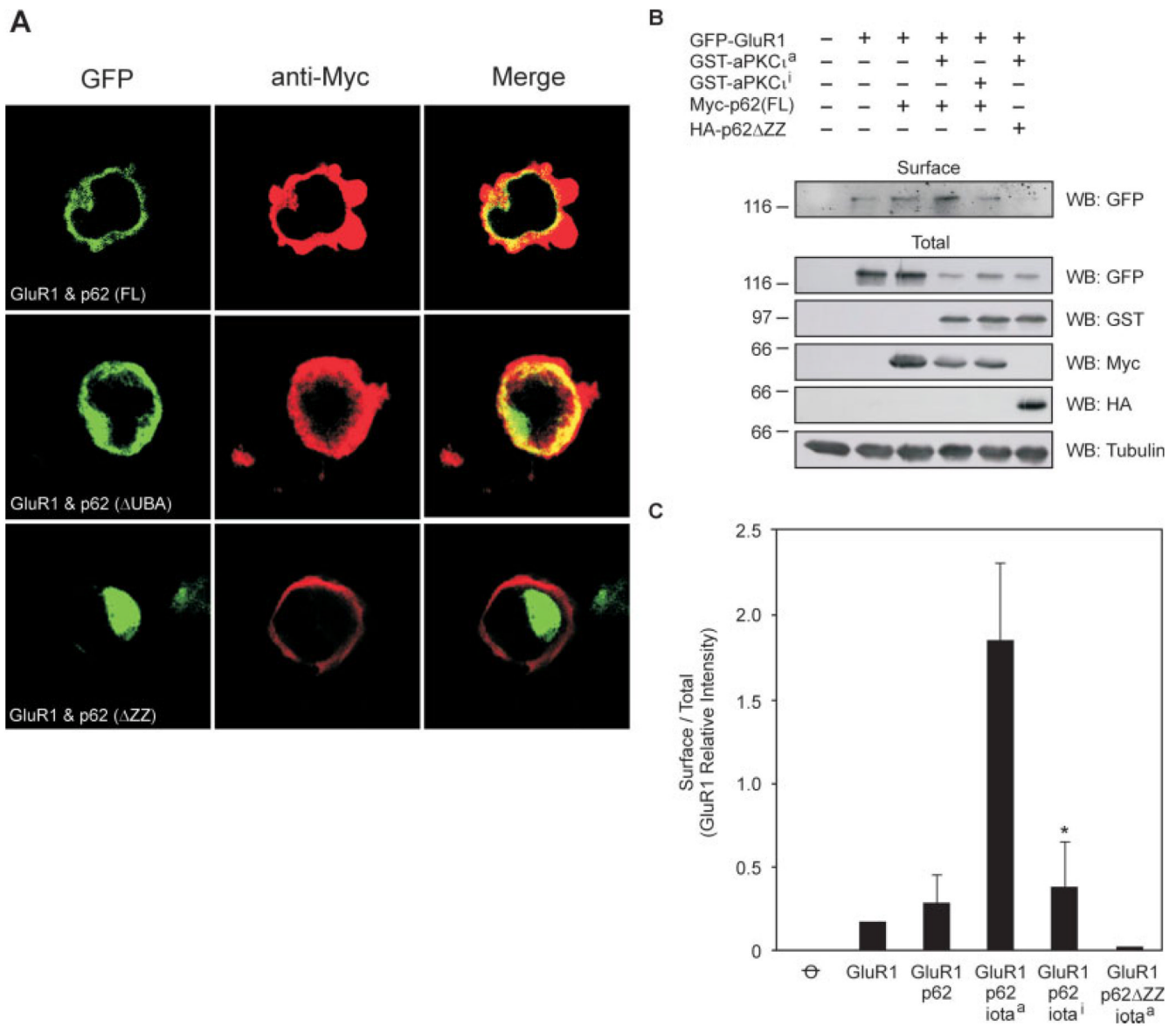


FIGURE 4.

ZZ-type Zinc finger domain of p62 is necessary for GluR1 recruitment to the cell surface. **A:** HEK cells were cotransfected with GFP-tagged GluR1 and Myc-tagged p62 (WT), p62 (ΔUBA), or p62 (ΔZZ). After 48 h, the cells were fixed, followed by addition of rabbit anti-Myc IgG and labeled with Texas Red-conjugated antirabbit antibodies (red). The colocalization of GluR1 and p62 (yellow) was examined by confocal microscopy. **B:** HEK cells were cotransfected as shown with GFP-GluR1, GST aPKC active (a)/inactive (i), Myc-tagged full length (FL) p62 and HA-tagged DZZ p62. The surface expression of GluR1 was examined by biotinylation. A fraction (50 μg) of the total lysate was blotted with antibodies to GFP, GST, Myc, HA, or tubulin as shown. **C:** The blots were scanned and the relative expression of GluR1 on the surface was normalized to the total GluR1 expressed in each sample ($X \pm S.E.M.$, $N = 3$); GluR1/p62/iota active > GluR1/p62/iota inactive (* $P < 0.05$). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

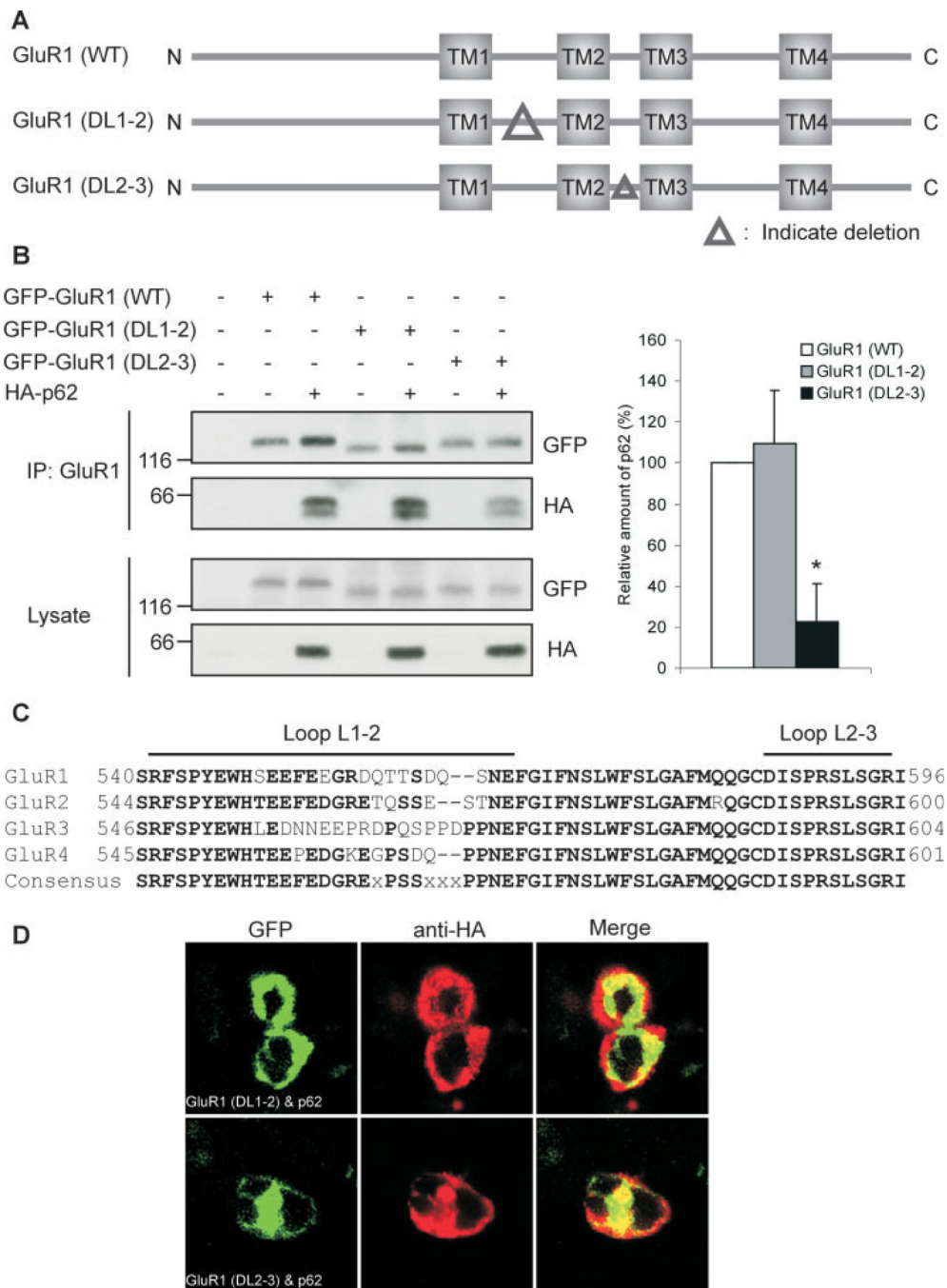


FIGURE 5. AMPA receptor subunits interact with p62 though the intracellular Loop L2–3. A: Schematic diagram of GFP-tagged GluR1 (WT), GluR1 (Δ L1–2), and GluR1 (Δ L2–3) mutant constructs. B: HEK cells were cotransfected with GFP-tagged GluR1 (WT), GluR1 (Δ L1–2), or GluR1 (Δ L2–3) and HA-tagged p62. After 48 h, the cells were harvested and lysed with Triton Lysis buffer. The cell lysates (750 μ g) were immunoprecipitated with anti-GluR1, then Western blotted with anti-GluR1 and anti-HA (Left upper 2 panels). The lysates (50 μ g) also were Western blotted with anti-GFP and anti-HA to verify expression of recombinant constructs. (Right) The relative interaction of p62 with GluR1 mutants was examined by coimmunoprecipitation and the resulting blots scanned. The experiment was repeated four

independent times. The data represent the mean \pm SEM (* $N = 4$, $P < 0.005$). C: Intracellular loop sequence alignment of AMPA receptor subunits GluR1–4. D: HEK cells were cotransfected with either GFP-tagged GluR1 (Δ L1–2) or GluR1 (Δ L2–3) and HA-tagged p62. After 48 h, the cells were fixed, followed by incubation with rabbit anti-HA IgG, and labeled with Texas Red conjugated antirabbit antibodies (red). The colocalization (yellow) was examined by confocal microscopy. GluR1 (Δ L1–2) colocalized with p62 in the cell membrane (yellow); whereas the deletion of intracellular loop L2–3 significantly reduced the colocalization of GluR1 and p62 at the cell membrane. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

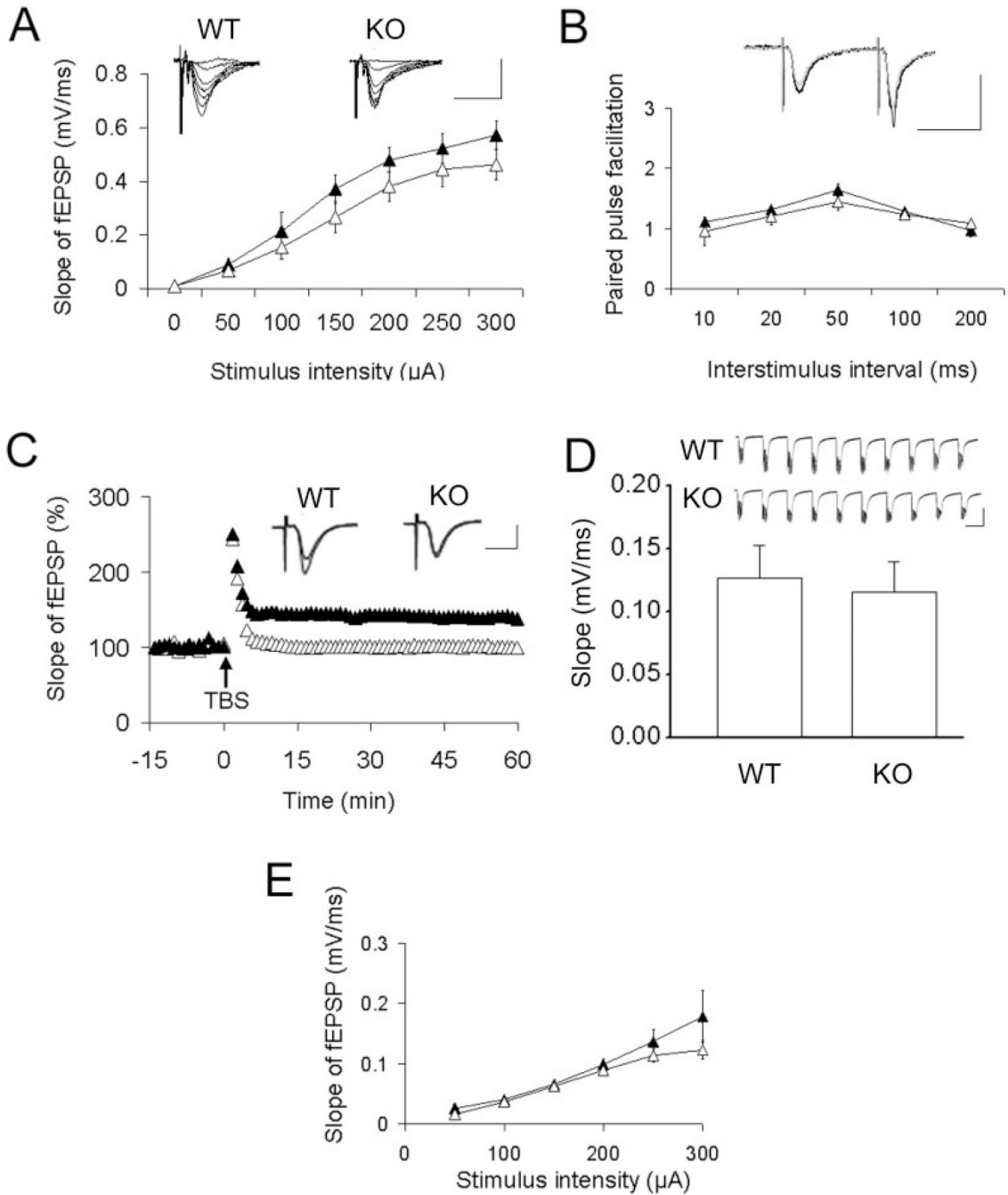
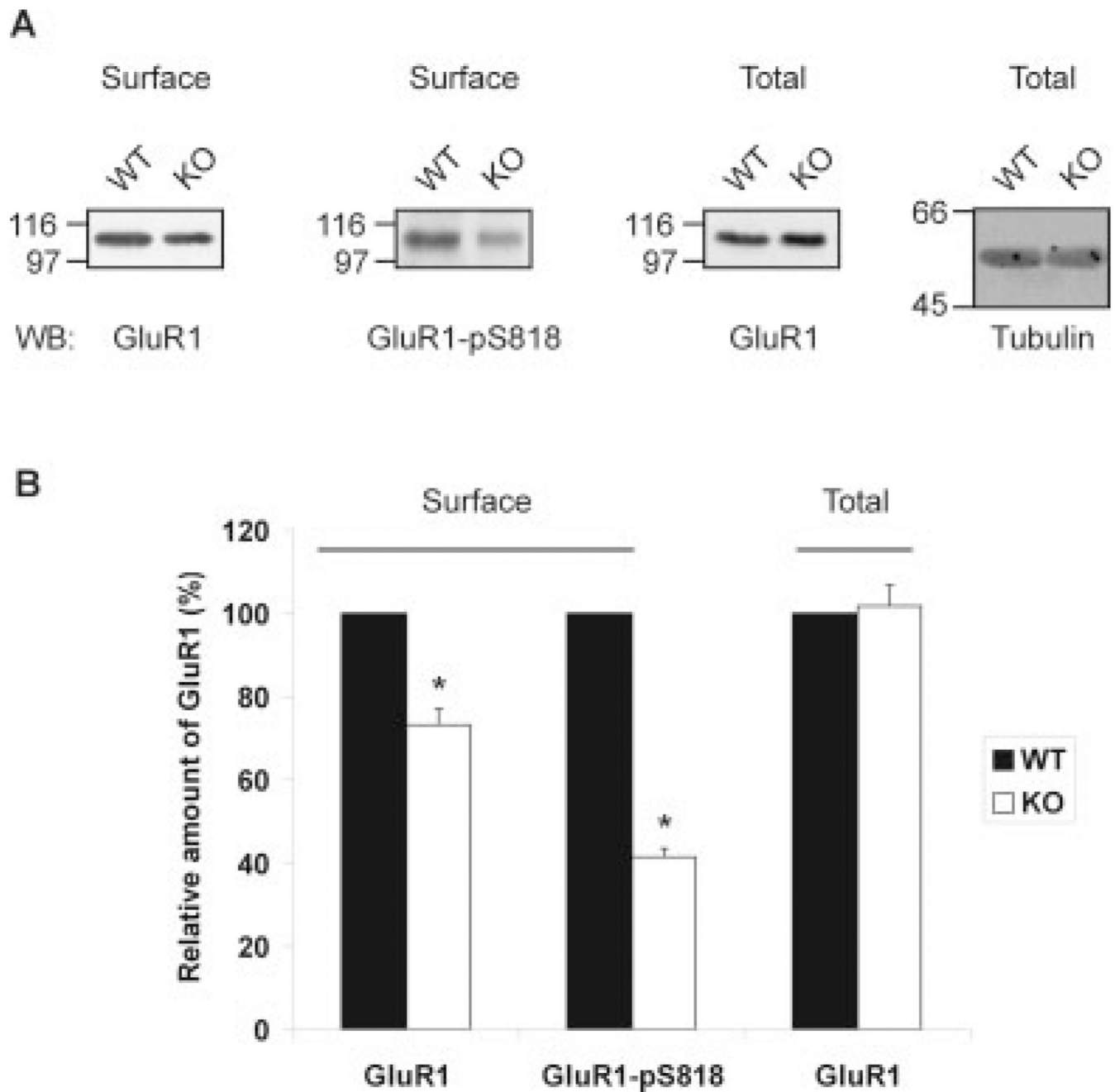


FIGURE 6. Basal synaptic transmission and plasticity in hippocampal Shaffer collateral-CA1 synapses. A: Input/output curves and representative fEPSPs at increasing stimulus strength showed no differences between wild-type (WT) and p62 knock-out (KO) mice. Calibration: 1.5 mV, 20 ms. For each plot representative fEPSPs of p62 (-/-) animals are shown on right to that of WT. B: PPF showed no significant differences between the two groups of mice. Overlaid representative fEPSPs (black WT; gray KO) have 50 ms interstimulus interval. Calibration: 1.5 mV, 20 ms. C: Synaptic plasticity (long term potentiation, LTP) was induced by TBS and measured for 50–60 min after TBS. Compared with WT mice, the LTP was abolished in p62 KO mice; whereas post tetanic potentiation (<2 min post TBS) in the KO remained normal.

Traces show averages of 30 fEPSPs recorded before TBS overlaid with averages of 30 fEPSPs recorded 50 min after TBS. Calibration: 1 mV, 15 ms. D: Top panel: The fEPSPs evoked by the first TBS in WT and KO mice were similar. Calibration: 0.5 mV and 150 ms. The integral associated with the TBS wave form in KO vs. WT were calculated and group means were compared by analysis of variance (ANOVA). There was no significant difference between p62 WT and KO data ($P < 0.05$). E: The input output curves of NMDAR mediated fEPSPs did not show any significant difference between WT and KO. In all plots, WT data are represented by the filled triangles and KO data are represented by open triangles. The data are represented as $X \pm S.E.M$ ($P < 0.05$). Each experimental group contained recordings from 5 to 8 animals, 2 slices per animal.

**FIGURE 7.**

p62 facilitates AMPA receptor surface delivery. A: Acute hippocampal slices from wild-type (WT) and p62 knock-out (KO) mice were biotinylated. The surface and total protein were separated by SDS-PAGE and Western blotted for GluR1, GluR1-pS818, and tubulin. B: The relative amount of GluR1 and GluR1-pS818 at the surface or total was determined by densitometric scan of the blots ($n = 3$). The relative differences $X \pm SEM$ were tested. $*P < 0.05$.

A

		<u>p62 interacting site</u>		
GABA _C -p3	419	QIKRKS	SLGGN	428
GluR1	586	DISPR	SLSGR	595
Grb14	370	SIEN	SLVAM	379
RIP	368	EENE	PSLQSK	377
Consensus		ISE_xSL		

B

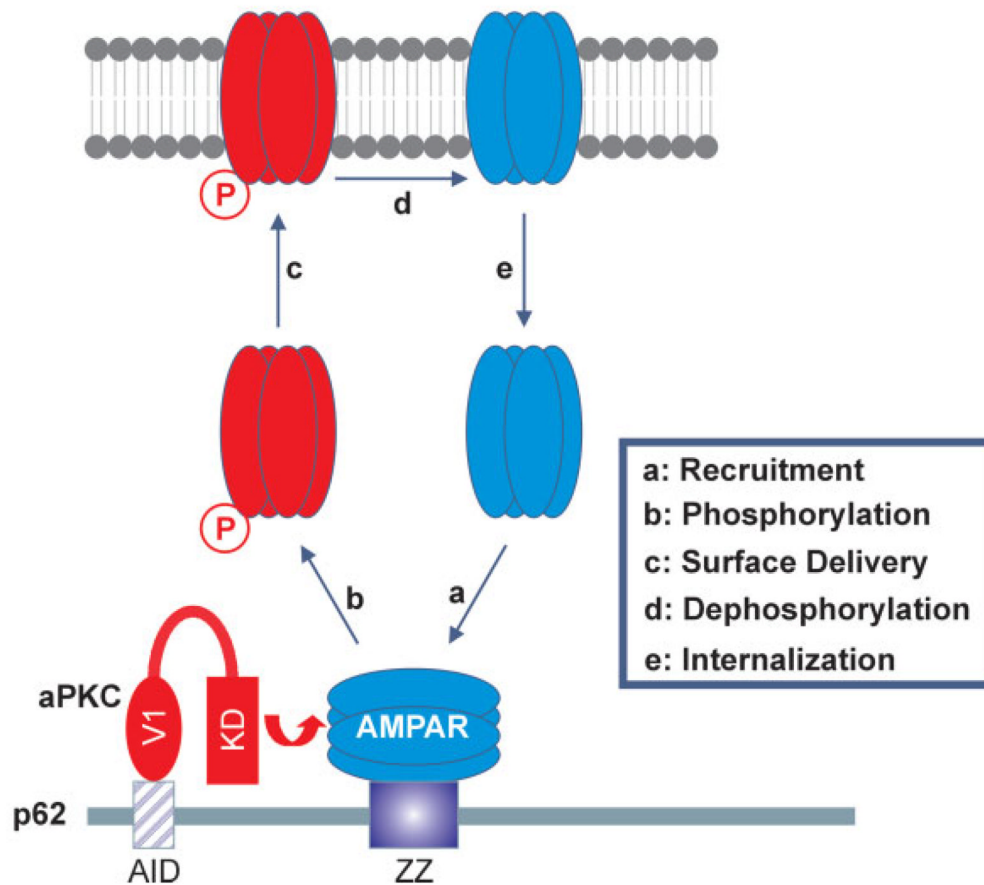


FIGURE 8.

p62 serves as a scaffold. A: Alignment of the p62 interaction sites in AMPA receptor subunits GluR1–4, GABA_C receptor subunit p3, Grb14, and RIP indicates a conserved motif, ISE_xSL, shared by p62-interacting proteins. B: This model illustrates the role of p62 in synaptic targeting of AMPA receptor. p62 recruits AMPA receptor through its ZZ domain (a) for phosphorylation mediated by aPKC (b), then the phosphorylated AMPA receptor is delivered to the synaptic surface to mediate LTP (c). Once the receptor is dephosphorylated (d), the AMPA receptor is internalized (e), and can recycle back to the surface by phosphorylation (a). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Table 1
Mapping the Interaction of GluR1 with p62

										Interaction with GluR1						
p62 (FL)	1	50	66	82	128	163	225	251	266	294	345	377	386	434	440	+
	N	SH2		AID	ZZ		TRAF6	PEST		PEST	UBA			C		
p62 (ΔUBA)	N	SH2		AID	ZZ		TRAF6	PEST		PEST				C	+	
p62 (ΔN-term)							N	PEST		PEST	UBA			C	-	
p62 (1-266)	N	SH2		AID	ZZ		TRAF6		C							+
p62 (230-440)							N	PEST		PEST	UBA			C	-	
p62 (ΔTRAF6)	N	SH2		AID	ZZ		Δ	PEST		PEST	UBA			C	+	
p62 (ΔAID)	N	SH2		Δ	ZZ		TRAF6	PEST		PEST	UBA			C	+	
p62 (Δ82-122)	N	SH2		Δ	ZZ		TRAF6	PEST		PEST	UBA			C	+	
p62 (62-440)		N		AID	ZZ		TRAF6	PEST		PEST	UBA			C	+	
p62 (115-440)					N		ZZ	TRAF6		PEST	UBA			C	+	
p62 (ΔZZ)	N	SH2		AID	Δ		TRAF6	PEST		PEST	UBA			C	-	

Δ: Indicate deletion.

Table 2
Mapping the Interaction of p62 with GluR1






						Interaction with p62
GluR1 (WT)	N				C	+
GluR1 (1-875)	N				C	+
GluR1 (1-823)	N				C	+
GluR1 (1-812)	N				C	+
GluR1 (1-807)	N				C	+

Table 3
 Proteins Interacting with p62 Through ZZ-Type Zinc Finger Domain

	Binding site on protein	Role of p62	Reference
Receptor protein			
Kv β 2	Not mapped	Recruiting aPKC for phosphorylation	Gong et al., 1999
D ₂ DAR	Not mapped	Ubiquitination related	Kim, 2006
GABA _C - ρ 3	Intracellular loop (TM3–TM4)	Recruiting aPKC for phosphorylation	Croci et al., 2003
AMPA receptor	Intracellular loop (L2–3)	Trafficking	
Nonreceptor protein			
RIP	ID (intermediate domain)	Linking aPKC to NF- κ B activation	Sanz et al., 1999
Grb14	PIR (phosphorylated insulin receptor) domain	Recruiting aPKC for phosphorylation	Cariou et al., 2002