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Identification and characterization of a novel phosphorylation site on the GluR1 subunit of AMPA receptors

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Phosphorylation of various AMPA receptor subunits can alter synaptic transmission and plasticity at excitatory glutamatergic synapses in the central nervous system. Here, we identified threonine-840 (T840) on the GluR1 subunit of AMPA receptors as a novel phosphorylation site. T840 is phosphorylated by protein kinase C (PKC) *in vitro* and is a highly turned-over phosphorylation site in the hippocampus. Interestingly, the high basal phosphorylation of T840 in the hippocampus is maintained by a persistent activity of a protein kinase, which is counter-balanced by a basal protein phosphatase activity. To study the function of T840, we generated a line of mutant mice lacking this phosphorylation site using a gene knock-in technique. The mice generated lack T840, in addition to two previously identified phosphorylation sites S831 and S845. Using this mouse, we demonstrate that T840 may regulate synaptic plasticity in an age-dependent manner.

Introduction

Many of the brain functions critically depend on plasticity of the glutamatergic excitatory synaptic transmission, which can be accomplished postsynaptically by modulation of glutamate receptors. AMPA-type glutamate receptors mediate the majority of fast excitatory synaptic transmission, and functional changes to these receptors are implicated in synaptic plasticity (Song and Huganir, 2002; Collingridge et al., 2004). One way to alter the function of AMPA receptors is by changing phosphorylation of its subunits. All four subunits of AMPA receptors, GluR1–4, which have several identified phosphorylation sites on their intracellular carboxy-terminus (Song and Huganir, 2002).

Among the four subunits, GluR1 has three identified phosphorylation sites, serines 818 (S818) (Boehm et al., 2006), 831 (S831) and 845 (S845) (Roche et al., 1996). S831 is phosphorylated by calcium/calmodulin-dependent protein kinase II (CaMKII) and protein kinase C (PKC), S845 is a cAMP-dependent protein kinase (PKA) substrate (Roche et al., 1996; Barria et al., 1997a; Mammen et al., 1997), while S818 is phosphorylated by PKC (Boehm et al., 2006). Changes in phosphorylation of GluR1 at S845 and S831 affect AMPA receptor mediated currents (Derkach et al., 1999; Banke et al., 2000) and are involved in long-term potentiation (LTP) and long-term depression (LTD) in the hippocampus (Barria et al., 1997b; Kameyama et al., 1998; Lee et al., 1998, 2000, 2003). Recent data suggest that S845 and S818 affect the trafficking AMPA receptors to synapses and/or stabilize synaptic AMPA receptors (Esteban et al., 2003; Lee et al., 2003; Boehm et al., 2006).

Phosphorylation of GluR2 subunit at serine-880 has been implicated in LTD both in hippocampus and in cerebellum (Daw et al., 2000; Matsuda et al., 2000; Xia et al., 2000; Kim et al., 2001; Chung et al., 2003). Phosphorylation of this site can regulate synaptic localization of AMPA receptors by altering its interaction with intracellular binding partners: glutamate receptor interacting protein (GRIP)/AMPA receptor binding protein (ABP) and protein interacting with C-kinase-1 (PICK-1) (Matsuda et al., 1999; Chung et al., 2000).

Among several identified GluR4 subunit phosphorylation sites (Carvalho et al., 1999), S842, which is a PKA phosphorylation site, was shown to be involved in activity-dependent trafficking of AMPA receptors to synapses (Esteban et al., 2003). Therefore, phosphorylation of different subunits of AMPA receptor seems to play an important role in regulating receptor function, which can alter synaptic transmission and/or synaptic plasticity.

We had recognized early on from generating phosphopeptide maps of the GluR1 subunit that there remain additional unidentified phosphorylation sites on GluR1 (Blackstone et al., 1994; Roche et al., 1996; Mammen et al., 1997). Here, we report that threonine-

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840 (T840) is one of the major phosphorylation sites on GluR1. This site is phosphorylated by PKC *in vitro* and can be regulated by altering PKC activity in hippocampal slices. Interestingly, phosphorylation of T840 undergoes rapid turnover under basal conditions in the hippocampus, sustained by a balance between persistently active protein kinase(s) and protein phosphatase(s). To study the functional role of GluR1 T840, we generated a line of mice lacking T840 and show that it may be involved in regulating synaptic plasticity mechanisms in an age-dependent fashion.

Results

Mapping of a "basal" phosphorylation site on GluR1

To identify phosphorylation sites on GluR1 subunit of AMPA receptors, we generated phosphopeptide maps using trypsin digested GluR1 immunoprecipitated from metabolically labeled hippocampal slices. We were specifically interested in a large phosphopeptide spot (#1 in Fig. 1) that had not been previously characterized. This phosphopeptide was highly phosphorylated under basal conditions and was not noticeably upregulated by either forskolin (50 μM, 30 min) or phorbol ester (TPA: 1 μM, 30 min) treatment to the slices. Treatment of forskolin to slices upregulated phosphorylation on a phosphopeptide (#5 in Fig. 1) that contains S845, a previously identified PKA phosphorylation site (Roche et al., 1996), without affecting much the other phosphopeptides. On the other hand, phorbol ester treatment increased the signal of two phosphopeptides (#3 and #6 in Fig. 1), which contain S831, a known CaMKII and PKC phosphorylation site (Roche et al., 1996; Mammen et al., 1997).

To identify the phosphorylation site responsible for the phosphorylation of phosphopeptide #1, we transfected HEK293 cells with various GluR1 constructs. Transfection of a wildtype GluR1 generated a similar phosphopeptide map to the one obtained from GluR1 immunoprecipitated from hippocampal slices (Fig. 1B, left panel). We identified T840 as a responsible phosphorylation site since mutation of T840 to an alanine (GluR1-T840A) eliminated phosphopeptide #1 (Fig. 1B, middle panel). As shown in Fig. 1, phosphopeptide #1 migrates in close proximity to phosphopeptide #6, which includes S831. Therefore, we further confirmed that T840A indeed eliminates phosphopeptide #1 by generating a phosphopeptide map using GluR1 with both T840A and S831A mutations (Fig. 1B, right panel). In addition to phosphopeptide #1, phosphopeptides #3 and #6 corresponding to S831 were also absent. Our results indicate that T840 site is responsible for the phosphorylation of phosphopeptide #1.

Generation of T840 phosphospecific antibody

In order to characterize the role of GluR1-T840 phosphorylation site, we generated phosphorylation site-specific antibody (Ab)

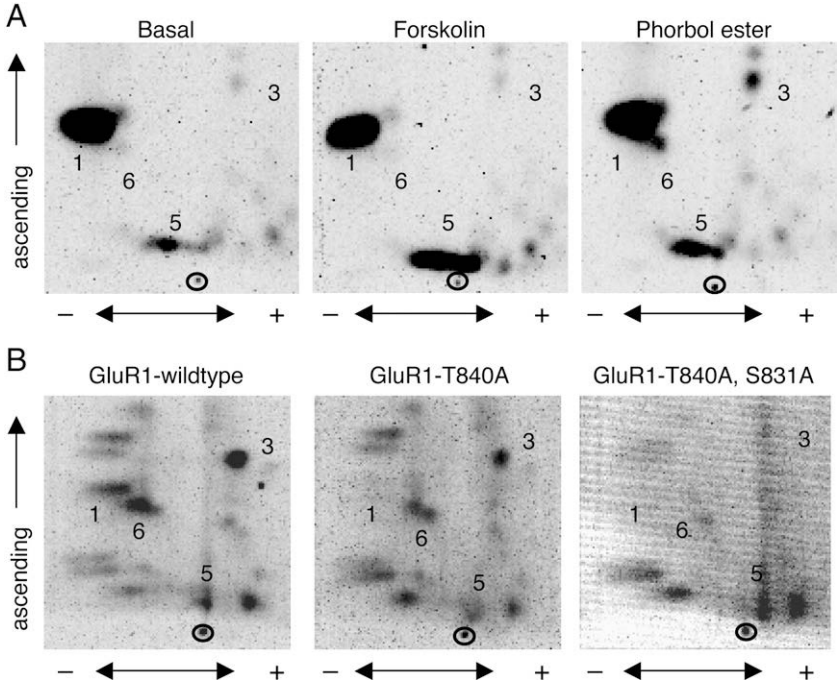

Fig. 1. Identification of T840 phosphorylation site. (A) Phosphopeptide map of GluR1 isolated from metabolically labeled hippocampal slices. Left panel shows phosphopeptide map of GluR1 from hippocampal slices under basal conditions. The phosphopeptide numbers are assigned following the conventions used in previous studies (Blackstone et al., 1994; Roche et al., 1996; Mammen et al., 1997). Note that the phosphopeptide #1 is highly phosphorylated under basal conditions. Middle panel shows GluR1 phosphopeptide map from forskolin (50 μM, 30 min) treated hippocampal slices. Forskolin treatment increased phosphorylation of phosphopeptide #5, which contains a previously identified PKA phosphorylation site S845. Right panel shows GluR1 phosphopeptide map from hippocampal slices treated with phorbol ester (1 μM, 30 min). There is an increase in phosphorylation of phosphopeptides #3 and #6, which contain S831, a site phosphorylated by CaMKII and PKC. Circle at the bottom depicts the origin. Double-headed arrow at the bottom illustrates the direction of peptide separation by electrophoresis, and the arrow on the side shows the direction of separation by chromatography. (B) Phosphopeptide map of GluR1 isolated from metabolically labeled HEK293 cells transfected with various GluR1 cDNA constructs. Left panel displays phosphopeptide map of wildtype GluR1. Phosphopeptides #1, #3, #5 and #6, which are present in GluR1 isolated from hippocampal slices (shown in A) are also present. Note that the intensity of phosphopeptide #1 is less than that in hippocampals slices. Middle panel shows GluR1 with T840 mutated to alanine (T840A). Phosphopeptide #1 is absent in the protein produced by the GluR1-T840A construct. Right panel shows phosphopeptide map of GluR1 with T840A and S831A mutations. Here, phosphopeptides #1, #3 and #6 are absent.

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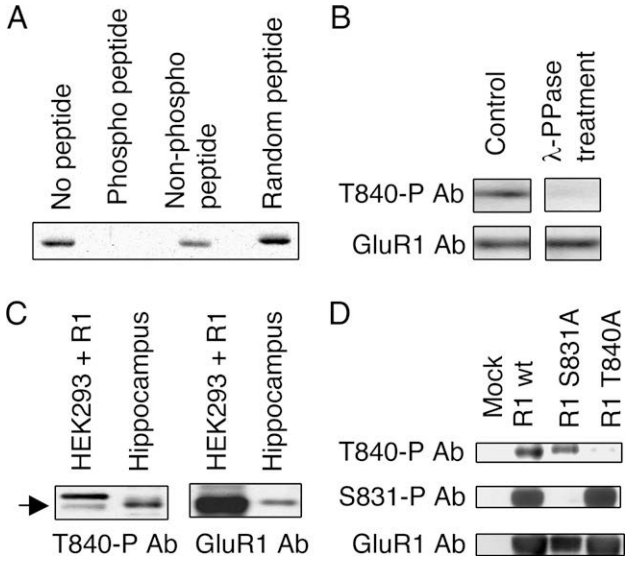

Fig. 2. Generation of T840 phosphorylation site-specific antibody. (A) T840 phospho-antibody signal is blocked specifically when the antibody was preincubated with the antigen phosphopeptide (lane 2), while the signal is intact when preincubated with the same sequence non-phosphorylated peptide (lane 3) or random sequenced peptide (lane 4). (B) Signal generated by T840 phospho-antibody disappears when hippocampal samples were preincubated with lambda phosphatase (top panel). Note that GluR1 C-terminus recognizing antibody still recognized GluR1 in lambda phosphatase treated samples (bottom panel). (C) Comparison of GluR1-T840 phosphorylation level in membrane (P2) fractions prepared from HEK293 cells expressing GluR1 and hippocampal slices. Note that T840 phosphorylation level in HEK293 cells is much less compared to that in hippocampus (left panel) despite the higher expression of GluR1 in HEK293 cells (shown by probing with GluR1 C-Ab; right panel). (D) T840 phospho-antibody specifically recognizes T840 phosphorylation site. HEK293 cells were transfected with cDNAs of wildtype GluR1, GluR1 with S831A mutation, and GluR1 with T840A mutation. T840 phospho-antibody signal disappeared in cells transfected with GluR1-T840A construct (top blot, lane 4), while S831 phospho-antibody signal is absent in cells transfected with GluR1-S831A construct (middle blot, lane 3). Expression levels of each GluR1 constructs were equivalent as shown using GluR1 C-terminal antibody (bottom blot).

against T840. The T840 phospho-Ab was specific to phosphorylated T840 since preincubation with the antigen phosphopeptide blocked its signal while preincubation with the same sequence non-phosphopeptide or a random sequence peptide did not block the signal (Fig. 2A). To determine if the T840 phospho-Ab specifically recognizes phosphorylated GluR1, we performed immunoblots using hippocampal samples with or without lambda-phosphatase treatment. As shown in Fig. 2B, lambda-phosphatase treatment abolished the signal using GluR1-T840 phospho-Ab, but not with GluR1 C-terminal recognizing antibody.

The T840 phospho-Ab recognizes GluR1 in both hippocampal slices and in HEK293 cells transfected with GluR1 (Fig. 2C). However, the signal in HEK293 cells was much less compared to that obtained from hippocampal homogenates despite the fact that total GluR1 expression was much greater. This indicates that the basal phosphorylation level of T840 is higher in the hippocampus compared to that in transfected HEK293 cells. This is in line with our observation from the phosphopeptide maps where we saw a larger phosphorylation signal on phosphopeptide #1 in GluR1 isolated from hippocampal slices compared to that from transfected HEK293 cells (Fig. 1).

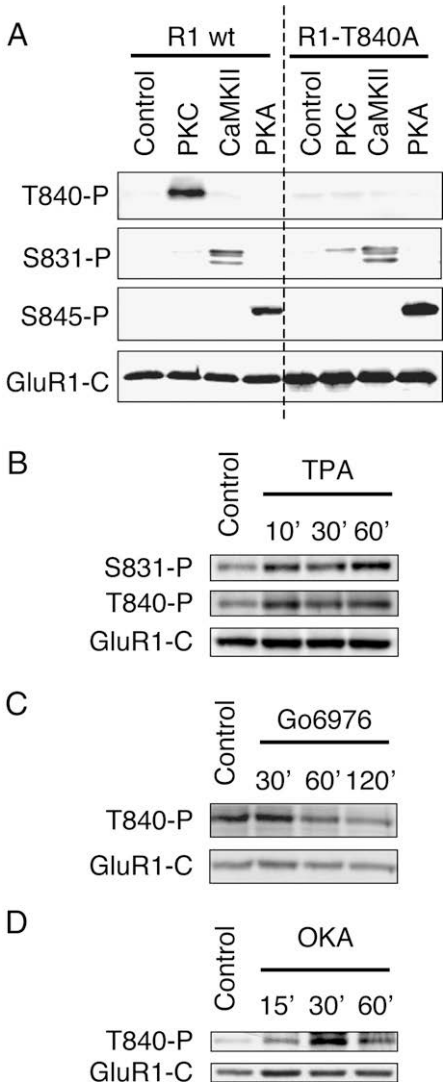

Fig. 3. GluR1-T840 is phosphorylated by PKC *in vitro* and is regulated by PKC and protein phosphatases in hippocampal slices. (A) *In vitro* phosphorylation assay using GST fusion proteins containing the last 80 amino acids of the GluR1 C tail and purified protein kinases. GluR1 wildtype fusion protein (R1 wt) incubated with PKC shows an increase in T840 phosphorylation (top blot, left panel) as detected by immunoblot analysis using T840 phospho-specific antibody. This increase was not seen when GluR1 fusion protein with T840A mutation (R1-T840A) was used (top blot, right panel). CaMKII specifically increased S831 phosphorylation (second blot from top), while PKA specifically increase S845 (third from top). PKC only marginally increased S831 phosphorylation, as detected by S831 phospho-antibody, under our conditions compared to CaMKII (second from top). Bottom blot was probed with GluR1 C-terminal recognizing antibody to show the amount of GluR1-C80 fusion protein used for each reaction. (B) Activation of PKC by phorbol ester (TPA, 1 μM) treatment to hippocampal slices increased both S831 (top blot) and T840 phosphorylation (middle blot). Bottom blot shows the GluR1 amount. (C) Blocking PKC activity by Go6976 (1 μM) treatment to hippocampal slices gradually decreased T840 phosphorylation (top blot). Total level of GluR1 is shown in the bottom blot probed with GluR1 C-terminal antibody. S831 phosphorylation did not decrease with Go6976 in the time course used (data not shown), suggesting that this phosphorylation site may turnover slower than T840. (D) Blocking protein phosphatase by okadaic acid (OKA, 1 μM) treatment to hippocampal slices increased T840 phosphorylation (top blot). Bottom blot shows the GluR1 total level.

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To further examine the specificity of the T840 phospho-Ab, we tested it against various mutations of GluR1 expressed in HEK293 cells. Mutation at T840A eliminated the signal using T840 phospho-Ab, while mutation at S831A abolishes signal using S831 phospho-Ab but not with T840 phospho-Ab (Fig. 2D).

T840 is phosphorylated by PKC in vitro and is regulated by PKC and protein phosphatases in hippocampal slices

Next we wanted to determine the protein kinase responsible for the phosphorylation of GluR1-T840 site. To do this we first performed an *in vitro* phosphorylation assay using GluR1 C-terminal GST fusion proteins and purified protein kinases. Phosphorylation of wildtype GluR1 fusion proteins with PKC increased T840 phosphorylation detected using T840 phospho-antibody, while CaMKII and PKA did not (Fig. 3A). Mutating T840 to an alanine in the GluR1 fusion protein (R1-T840A) abolished the phosphorylation by PKC, further confirming that PKC was affecting T840 phosphorylation (Fig. 3A). CaMKII and PKA were active in our assay since they were able to phosphorylate their respective substrates, S831 and S845 (Fig. 3A).

To examine whether PKC activation is responsible for T840 phosphorylation *in vivo*, we treated hippocampal slices with a PKC activator phorbol ester (TPA, 1 μM). As shown in Fig. 2B, TPA treatment increased T840 phosphorylation in parallel to S831, a previously identified PKC site. Since T840 phosphorylation is basally high in hippocampal slices, we next examined if an ongoing PKC activity is required to maintain this. We tested this by treating hippocampal slices with a PKC inhibitor, Go6976 (1 μM). We found that T840 phosphorylation was decreased by Go6976

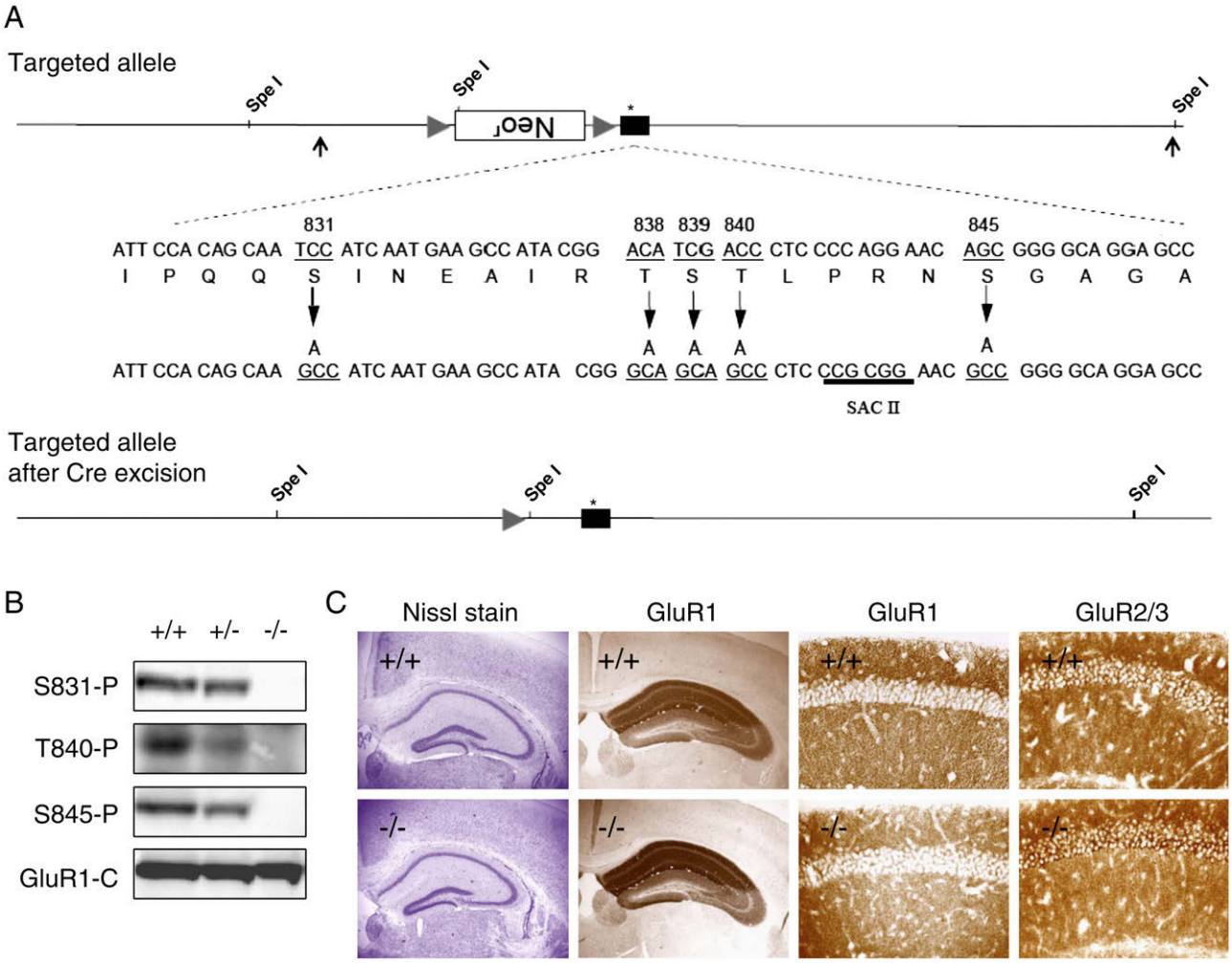

Fig. 4. Generation of GluR1 "Penta" phosphomutant mouse. (A) Schematic representation of GluR1 "penta" phosphomutant mouse. Target allele is indicated (top panel). The homologous region of genomic DNA used for targeting vector is indicated by arrows. Neo^r cassette with loxP sequences at both sides (short arrows) was introduced in the intron upstream of the last coding exon of GluR1. After germline transmission, Neo^r cassette was deleted using Cre-loxP system by breeding to CMV-Cre transgenic mouse (bottom panel). The introduced alanine substitutions (*) into phosphorylation sites and *Sac*II site were shown (middle). (B) Immunoblot showing the lack of S831, T840 and S845 phosphorylation sites on GluR1 from hippocampal membrane (P2) preparation of wildtype (+/+), heterozygote (+/−) and homozygote (−/−) "penta" mice. Note that GluR1 is still expressed in the homozygotes (−/−), as shown using GluR1 C-terminal recognizing antibody (bottom panel). (C) Distribution of GluR1 in "penta" mice. Normal gross anatomy shown by Nissl staining in hippocampal section in wildtype (Wt) and "penta" phosphorylation mutant mice (−/−) (left panel). Similar GluR1 localization in hippocampus shown by immunohistochemical staining using GluR1-C antibody (low magnification: second panel from the left, high magnification: third panel from the left). High magnification of GluR2/3-C antibody staining in CA1 hippocampus (far right panel).

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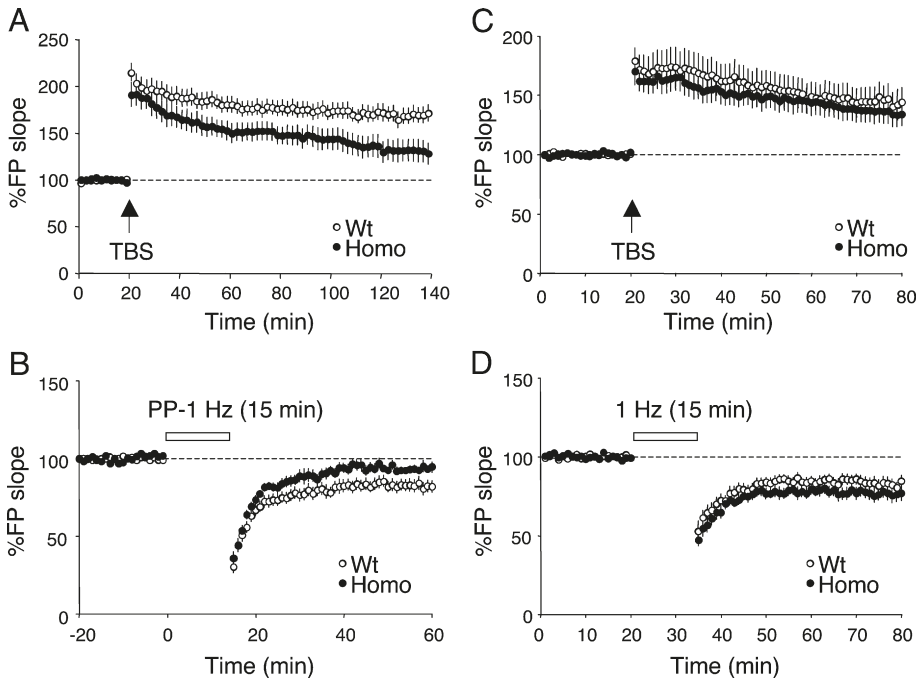

Fig. 5. Synaptic plasticity in adult (>3 months old) and young (3–4 weeks old) "penta" phosphomutants and wildtype littermates. (A) LTP induced by theta burst stimulation (TBS) is reduced in adult homozygotes (closed circles) compared to wildtype littermates (open circles). Note a faster decay of LTP in the homozygotes. (B) LTD induced by paired-pulse 1 Hz (PP-1 Hz, 15 min, ISI=50 ms) protocol is absent in adult homozygotes (closed circles) compared to wildtype littermates (open circles). (C) LTP induced by TBS is normal in young homozygotes (closed circles) compared to wildtype littermates (open circles). (D) LTD induced by 1 Hz (15 min) protocol is similar in young homozygotes (closed circles) and wildtype littermates (open circles).

treatment, which indicates that a persistent PKC activity is needed to keep the basal phosphorylation at T840. This suggests that the basal level of T840 phosphorylation should be susceptible to alterations in basal protein phosphatase activity. To test this we incubated slices with a broad spectrum protein phosphatase inhibitor okadaic acid (1 μM). As shown, okadaic acid treatment enhanced phosphorylation of T840 from early time points (Fig. 3D). This is in contrast with S831 and S845, where phosphorylation detectably increases only after incubation in okadaic acid for more than 2 h (Kameyama et al., 1998) (data not shown). Collectively, our results suggest that the high basal phosphorylation of GluR1-T840 in hippocampal slices is maintained by a persistent activity of PKC, which is counter-balanced by a basal protein phosphatase activity.

Generation of a GluR1 "penta" phosphorylation site mutant that lacks GluR1-T840

In order to test the *in vivo* function of GluR1-T840 phosphorylation site, we wanted to generate mice lacking T840 on GluR1 using a gene knock-in technique. We have previously characterized a line of mice that lack previously identified phosphorylation sites S831 and S845 (Lee et al., 2003). LTP was reduced while LTD was abolished in this "double" phosphomutants. Our initial question was whether the residual LTP could be due to T840 phosphorylation. To test this hypothesis, we generated mice lacking S831, S845 and T840 phosphorylation sites. This was accomplished by combining the following mutations on the GluR1 target construct: S831A, T838A, S839A, T840A and S845A (Fig. 4A). This "penta" mutation was necessary since mutating T840A alone might allow the responsible protein kinase to phosphorylate upstream T838 or S839, which might substitute the function of T840.

We confirmed the mutations in the knock-in mice using both PCR and immunoblot analysis (Figs. 4A and B). The mutants did not show any gross abnormalities in anatomy and behavior. Hippocampal cytoarchitecture was normal as shown using Nissl stain, and GluR1 distribution was normal as shown using immunohistochemistry (Fig. 4C).

Deficits in LTP and LTD in adult "penta" phosphomutant mice

Next we tested synaptic plasticity in the CA1 region of hippocampal slices made from adult (2–3 months old) wildtype and homozygote littermates. LTP induced by a theta burst stimulation (TBS) was reduced in the homozygotes (128±11% of baseline measured at 2 h post-TBS, $n=11$, 7 mice) compared to the wildtypes (172±9% of baseline, $n=13$, 7 mice; Student's t-test: $P<0.01$; Fig. 5A). This result is similar to the previously described "double" phosphomutant, which only lacks S831 and S845, suggesting that T840 may not contribute additionally to LTP. Similar to the "double" phosphomutants, LTD induced with paired-pulse 1 Hz protocol (PP-1 Hz, paired pulses at interstimulus interval of 50 ms repeated at 1 Hz for 15 min) was abolished in the "penta" homozygotes (94±3% of baseline at 1 h post-onset of PP-1 Hz, $n=12$, 6 mice) compared to wildtypes (82±4% of baseline, $n=11$, 5 mice; Student's t-test: $P<0.02$; Fig. 5B).

Normal LTP and LTD in young "penta" phosphomutants

There is now accumulating evidence that molecular mechanisms of LTP and LTD may differ in young versus adult hippocampus

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(Zamanillo et al., 1999; Jensen et al., 2003; Lee et al., 2003; Wikstrom et al., 2003; Yasuda et al., 2003; Palmer et al., 2004; Nosyreva and Huber, 2005). In particular, the involvement of GluR1 in LTP has been reported to occur only in adults (Zamanillo et al., 1999; Jensen et al., 2003). Similarly, we have reported using the "double" phosphomutants that LTP is normal in young mutants despite a deficit seen in adults (Lee et al., 2003). Therefore, we next looked at LTP and LTD in young "penta" phosphomutants. We found that LTP is normal in the young "penta" mutants (homozygotes: 138±9% of baseline, $n=16$, 6 mice; wildtype: 149±12% of baseline, $n=15$, 7 mice; Fig. 5C). In addition, LTD induced with 1 Hz stimulation was normal in the young "penta" mutants (homozygotes: 77±5% of baseline, $n=10$, 6 mice; wildtype: 85±3% of baseline, $n=12$, 6 mice; Fig. 5C). This result is different from our previous observation from "double" mutants, where LTD was absent in the young homozygotes (Lee et al., 2003).

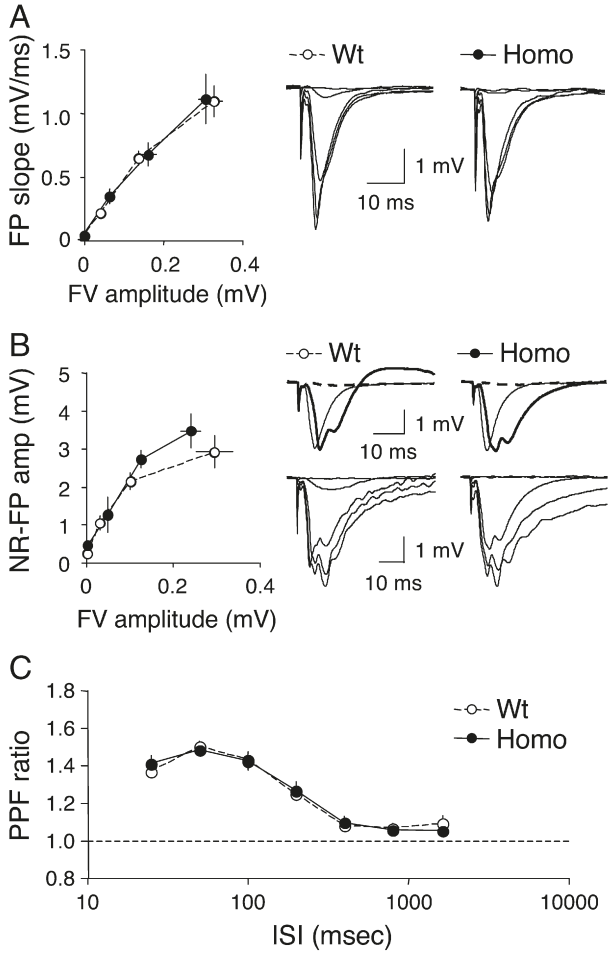

Fig. 6. Normal basal synaptic transmission in "penta" phosphomutants. (A) Basal synaptic transmission mediated mainly by AMPA receptors was normal in adult "penta" phosphomutants (closed circles; $n=30$, 10 mice) compared to wildtype littermates (open circles; $n=33$, 11 mice). Input–output function was generated by gradually increasing the stimulus intensity. The resulting field potential (FP) slope was plotted against the presynaptic fiber volley amplitude. (B) Pharmacologically isolated NMDA receptor-mediated synaptic responses were normal in adult "penta" phosphomutants (closed circles; $n=6$, 2 mice) compared to wildtype littermates (open circles; $n=6$, 2 mice). Pharmacologically isolated NMDA receptor-mediated field potential amplitude was plotted against the presynaptic fiber volley amplitude. (C) Paired-pulse facilitation (PPF) ratios measured across different interstimulus intervals (ISIs) are equivalent between adult "penta" mutants (closed circles; $n=8$, 3 mice) and wildtype littermates (open circles; $n=8$, 3 mice).

Normal basal synaptic transmission in adult "penta" phosphomutants

To rule out the possibility that the observed deficits in LTP and LTD in the adult "penta" mice were due to abnormalities in basal synaptic function, we compared basal synaptic transmission between homozygotes and wildtype littermates. Basal AMPA receptor-mediated synaptic responses and pharmacologically isolated NMDA receptor-mediated synaptic responses were both normal in the "penta" mutants (Figs. 6A and B). In addition, presynaptic function assessed by measuring the paired-pulse facilitation (PPF) ratio at various interstimulus intervals (ISIs) did not differ between the "penta" homozygotes and wildtypes (Fig. 6C). Therefore, lacking T840, in addition to S831 and S845, does not seem to alter basal synaptic transmission measured using our assays.

Discussion

Here we identified and described T840 as a novel phosphorylation site on GluR1 subunit of AMPA receptors. GluR1-T840 is phosphorylated by PKC *in vitro* and is a highly turned-over phosphorylation site under basal conditions in the hippocampus. The basal phosphorylation of GluR1-T840 *in vivo* is likely due to a persistent activity of a protein kinase, which is counter-balanced by a basal protein phosphatase activity. In order to test the *in vivo* role of GluR1-T840, we generated a GluR1 "penta" phosphomutant, which lacks not only T840 but also S831 and S845, two previously characterized phosphorylation sites (Roche et al., 1996; Mammen et al., 1997). We found that adult "penta" mice show similar phenotypes as the previously described "double" phosphomutants lacking only S831 and S845 phosphorylation sites, in that they show deficits in both LTP and LTD (Lee et al., 2003). However, in the young "penta" mice, LTP and LTD were both intact. This is different from the "double" mutants, which show normal LTP but have deficits in LTD (Lee et al., 2003).

Our results show that GluR1-T840 is highly phosphorylated under basal conditions in the hippocampus compared to GluR1 transfected HEK293 cells. This could be interpreted that the activity of protein kinase(s) responsible for T840 phosphorylation is higher in the hippocampus. Alternatively, the protein kinase responsible for the phosphorylation of T840 may not be present in the HEK293 cells. Although our *in vitro* phosphorylation data suggest that PKC can phosphorylate T840, whether PKC is indeed the protein kinase responsible for *in vivo* phosphorylation is unclear. Our data indicate that the protein kinase responsible for phosphorylating T840 *in vivo* should be affected by PKC activator phorbol ester and inhibitor Go6976 (Fig. 3), either because the drugs directly affect its activity or indirectly through alterations in PKC activity. In any case, our results indicate that the activity of this protein kinase is likely to be high in the hippocampus under basal conditions.

An interesting finding is that the basal phosphorylation of T840 seems to undergo a rapid turnover, which is due to persistent activity of a protein kinase and a protein phosphatase. This is quite different from previously characterized S831 and S845 on GluR1. Blockade of basal protein phosphatase activity by okadaic acid

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quickly upregulated T840 (Fig. 3D), while S831 and S845 only increased with prolonged (>3 h) application (Kameyama et al., 1998). This suggests that S831 and S845 may not be readily accessible to protein phosphatases. In support of this the same PKC inhibitor (Go6976) that reduced T840 phosphorylation (Fig. 3C) did not affect S831 (data not shown). The mechanism in which protein phosphatases preferentially affect T840 is unclear at this point, but could be due to conformation of the GluR1 C-tail or by interacting proteins that mask the other sites.

To further elucidate the role of T840 phosphorylation site, we generated a line of "penta" phosphomutants that have alanine substitutions at five residues: S831A, T838A, S839A, T840A and S845A. We had reported previously that adult "double" phosphomutant mice (S831A and S845A) display residual LTP (Lee et al., 2003). Therefore, we wanted to test whether the remaining T840 could have contributed to the phenotype. Hence our strategy was to mutate T840 in addition to the two previously characterized phosphorylation sites. Our assumption was that several phosphorylation sites may interact with each other to regulate GluR1 function, which is in line with a recent study (Boehm et al., 2006). Despite the fact that the basal phosphorylation of GluR1-T840 is high in the hippocampus, we did not observe a difference in basal AMPA receptor-mediated synaptic transmission in our "penta" mutants. Therefore, the function of the GluR1-T840 is not likely to maintain basal synaptic transmission. In addition, it is also unlikely that GluR1-T840 participates in LTP or LTD in the adult hippocampal CA1 region since the phenotype we saw is similar to our "double" phosphomutants lacking only S831 and S845 (Lee et al., 2003). An interesting observation that may lead to the function of GluR1-T840 comes from our data using young "penta" mice. We found that both LTP and LTD are intact in the young "penta" mice. This is qualitatively different from the "double" mutants, where LTP was intact but LTD was defective (Lee et al., 2003). This suggests that the lack of T840 can somehow "rescue" LTD in young mice when both S831 and S845 phosphorylation sites are missing. Whether this "rescue" is due specifically to lacking T840 in addition to S831 and S845 or due to the additional mutations at T838 and S839 cannot be determined at this point. However, we surmise that the lack of T840 allows LTD to be expressed via another pathway not dependent on S831 and/or S845 phosphorylation site(s). One possibility is a recruitment of GluR2-dependent mechanisms for LTD (Daw et al., 2000; Kim et al., 2001; Seidenman et al., 2003). However, the link between GluR1-T840 and GluR2-dependent LTD mechanisms is presently not clear and awaits further exploration. The "rescue" of LTD by T840 mutation only occurred in young animals indicating that this putative alternative pathway for LTD is no longer accessible in the adults.

Experimental methods

Hippocampal slice preparation and metabolic labeling

Hippocampal slices from adult Sprague–Dawley rats (150–250 g, males) were prepared as previously described (Lee et al., 2000). In brief, 400 μm thick hippocampal slices were dissected using ice-cold dissection buffer (212.7 mM sucrose, 2.6 mM KCl, 1.23 mM NaH_2PO_4, 26 mM $NaHCO_3$, 10 mM dextrose, 3 mM $MgCl_2$ and 1 mM $CaCl_2$) and recovered at room temperature in ACSF (124 mM NaCl, 5 mM KCl, 1.25 mM NaH_2PO_4, 26 mM $NaHCO_3$, 10 mM dextrose, 1.5 mM $MgCl_2$ and 2.5 mM $CaCl_2$) inside a Plexiglas chamber supplied with 95% O_2/5% CO_2. For metabolic labeling, hippocampal slices were incubated with 1 mCi/ml [^{32}P]orthophosphate in ACSF for 2 h. Slices were then collected and quickly homogenized in 1 ml volume of ice-cold 1% Triton X-100 in IP buffer (10 mM $NaPO_4$, 150 mM NaCl, 5 mM EDTA, 5 mM EGTA, 10 mM sodium pyrophosphate, 50 mM NaF, 1 mM Na_3VO_4, 1 μM okadaic acid and 10 U/ml aprotinin, pH 7.4). Insoluble fraction was removed by centrifugation at 14,000×*g* for 10 min at 4 °C. The GluR1 subunit was immunoprecipitated using anti-GluR1 C-terminal antibody as described previously (Roche et al., 1996; Mammen et al., 1997). Samples were analyzed on a 7.5% SDS–PAGE gel and visualized by autoradiography.

Cell culture, transfection and metabolic labeling

HEK293T cells maintained at 37 °C and 5% CO_2 were transiently transfected with 10 μg of cDNA, using the calcium phosphate precipitation method, as previously described (Roche et al., 1996; Carvalho et al., 1999). Cells were used 48 h after transfection. For metabolic labeling, the cells were incubated with 1 mCi/ml [^{32}P]orthophosphate in phosphate-free minimal essential medium (Life Technologies) for 4 h. Cells were scraped in 150 μl of 1% SDS in IP buffer containing 10 mM sodium phosphate (pH 7.0), 100 mM NaCl and protease and phosphatase inhibitors. The cells were then diluted by adding 750 μl of 1% Triton X-100 in ice-cold IP buffer and sonicated. The residual insoluble fraction was removed by centrifugation at 14,000×*g* for 10 min at 4 °C. The GluR1 protein or GluR1 mutant proteins were isolated by immunoprecipitation, using anti-GluR1 C-terminus antibody, as described previously (Roche et al., 1996; Mammen et al., 1997). Samples were analyzed on a 7.5% SDS–PAGE gel and visualized by autoradiography.

Phosphopeptide mapping

Phosphopeptide mapping of phosphorylated proteins was performed essentially as previously described (Blackstone et al., 1994; Roche et al., 1996; Mammen et al., 1997). Briefly, the polyacrylamide gel fragments containing the phosphorylated GluR1 or GluR1 fusion proteins were excised from the gel, digested with 0.3 mg/ml trypsin and spotted onto TLC plates. The tryptic digests were separated by electrophoresis (400 V) in acetic acid/pyridine/H_2O (19:1:89, v/v) in the first dimension and by ascending chromatography in pyridine/butanol/acetic acid/H_2O (15:10:3:12, v/v) in the second dimension. The TLC plates were exposed in PhosphoImager cassettes and visualized with a PhosphoImager (Molecular Dynamics).

Generation of phosphorylation site-specific antibody

The peptide KEAIRTSTLPRN corresponding to amino acids 834–844 on GluR1 were synthesized (Middle Atlantic Mass Spectrometry Laboratory, Johns Hopkins University School of Medicine) with phosphothreonine included at the T840 position. Lysine residue was included to facilitate glutaraldehyde coupling to the carrier protein, thyroglobulin, and the resulting phosphopeptide/thyroglobulin mixtures were used to immunize New Zealand White rabbits. Sera were obtained periodically by Covance. Polyclonal antiphosphopeptide antibodies were purified from sera by sequential chromatographs on Affi-Gel 15 (Bio-Rad) columns covalently linked to phosphorylated and unphosphorylated peptides. Antibodies were first eluted from the phosphorylated peptide affinity columns using 100 mM glycine (pH 2.7). The affinity purified antibodies were dialyzed against phosphate-buffered saline (PBS, pH 7.4) and then loaded onto unphosphorylated peptide affinity columns. The flow-through was collected and used for immunoblot analysis.

In vitro phosphorylation assay of GST-fusion proteins

Phosphorylation of 1 μg of GST-fusion proteins of GluR1 C-terminal 80 amino acids was performed by incubation with 300 ng of purified kinases at 30 °C for 30 min in a 100 μl total volume. Phosphorylation reaction buffer for PKC and CaMKII reactions contained 100 mM HEPES, pH 7.5, 20 mM MgCl2, 200 μM CaCl2 and 250 μM ATP. For PKC reactions, 50 μg/ml phosphatidylserine and 5 μg/ml diacylglycerol were added. Phosphorylation of fusion proteins by PKA was performed in 20 mM

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HEPES, pH 7.0, 10 mM $MgCl_2$ and 250 μM ATP. Reactions were stopped with SDS–PAGE sample buffer, and the phosphorylated fusion proteins were resolved by SDS–PAGE and prepared for immunoblot analysis.

Generation of GluR1 "penta" phosphorylation site mutant mice

Gene targeting was carried out as reported previously (Lee et al., 2003). Serines 831, 839 and 845 and threonines 838 and 840 were mutated to alanine by site directed PCR mutagenesis (Stratagene). Correct homologous recombinant ES clones were injected into C57BL/6 blastocysts, and chimeras were crossed to C57BL/6 mice. These mice were crossed to CMV-Cre (129 and C57BL/6 hybrid genetic background) to remove the neomycin resistant gene (Neo^r) cassette from the germ line through Cre/loxP mediated excision (Nagy et al., 1998). After confirmation of Neo^r cassette excision by PCR and Southern blot analysis, heterozygous mice were bred to produce homozygotes, and mutants in which the Cre gene was absent were used for the following breeding. The intercross of heterozygotes resulted in production of wildtype, heterozygous and homozygous offspring at the expected 1:2:1 Mendelian ratio. For all analyses, mice with 129 and C57BL/6 hybrid genetic background were used. All procedure relating to animal care and treatment conformed to the institutional and NIH guidelines.

Hippocampal slice preparation and electrophysiology

Hippocampal slices from mice were prepared as previously described (Lee et al., 2003). In brief, 400 μm thick hippocampal slices were dissected using ice-cold dissection buffer and recovered at room temperature in ACSF bubbled with 95% O_2/5% CO_2. All recordings were done in a submersion recording chamber perfused with ACSF (29–30 °C, 2 ml/min) bubbled with 95% O_2/5% CO_2. Synaptic responses were digitized and stored online using Igor™ software (Wavemetrics). LTP was induced using a theta burst stimulation (TBS: four trains, each consisting of ten 100 Hz bursts (four pulses) given at 5 Hz, repeated at 10 s intervals) (Larson et al., 1986). LTD was elicited using a paired-pulse 1 Hz protocol (PP-1 Hz: paired-pulses of 50 ms interstimulus interval (ISI) repeated at 1 Hz for 15 min) (Lee et al., 2003) or a standard 1 Hz (15 min) protocol (Dudek and Bear, 1992). For measurement of paired-pulse facilitation (PPF), we used ISIs of 25, 50, 100, 200, 400, 800 and 1600 ms. Pharmacologically isolated NMDA receptor-mediated synaptic responses were measured using 0 mM $MgCl_2$ ACSF with 10 μM NBQX. At the end of each experiment, 100 μM D,L-APV was added to confirm the NMDA receptor-mediated response.

Immunoblot analysis

Proteins on SDS–PAGE gel were transferred to PVDF membranes (Immobilon™, Millipore), and the blots were blocked in blocking buffer (1% bovine serum albumin and 0.1% Tween 20 in PBS, pH 7.4) for ~1 h. The blots were then incubated for ~1 h in primary antibodies diluted in blocking buffer and washed 5×5 min with blocking buffer. Secondary antibody (for ECL: linked to horseradish peroxidase, for ECF: linked to alkaline phosphatase) diluted in blocking buffer was applied for ~1 h followed by 5×5 min washes with blocking buffer. The blots were developed using either enhanced chemiluminescence (ECL) or enhanced chemifluorescence (ECF, Amersham). ECL blots were exposed to BioMax film (Kodak) and digitized using a densitometer (Molecular Dynamics). ECF blots were scanned using a Storm 860 scanner (Molecular Dynamics), and the fluorescence intensity of each band was quantified using ImageQuant™ software (Molecular Dynamics).

Immunohistochemistry

Immunohistochemistry was performed as described (Lee et al., 2003). In brief, brains were fixed in 4% paraformaldehyde by transcardiac perfusion and transferred to a 30% sucrose solution to sink. Coronal sections were cut at 40 μm thickness on a freezing microtome (Leica) and collected into ice-cold 0.1 M PBS (pH 7.4). Sections were stained with cresyl violet and observed under the light microscope or processed for immunohistochemistry using anti-GluR1 C-terminal Ab or anti-GluR2 C-terminal Ab.

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