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Liprinα1 Degradation by Calcium/Calmodulin-Dependent Protein Kinase II Regulates LAR Receptor Tyrosine Phosphatase Distribution and Dendrite Development

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SUMMARY

Neural activity regulates dendrite and synapse development, but the underlying molecular mechanisms are unclear. Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) is an important sensor of synaptic activity, and the scaffold protein liprinα1 is involved in pre- and postsynaptic maturation. Here we show that synaptic activity can suppress liprinα1 protein level by two pathways: CaMKII-mediated degradation and the ubiquitin-proteasome system. In hippocampal neurons, liprinα1 mutants that are immune to CaMKII degradation impair dendrite arborization, reduce spine and synapse number, and inhibit dendritic targeting of receptor tyrosine phosphatase LAR, which is important for dendrite development. Thus, regulated degradation of liprinα1 is important for proper LAR receptor distribution, and could provide a mechanism for localized control of dendrite and synapse morphogenesis by activity and CaMKII.

INTRODUCTION

Activity-dependent calcium entry into neurons induces a variety of changes, ranging from transient posttranslational modifications of synaptic proteins to altered gene expression. At excitatory synapses, Ca^{2+} influx through voltage-gated Ca^{2+} channels and ionotropic glutamate receptors (particularly NMDA receptors) triggers biochemical cascades that regulate synaptic function (Kennedy et al., 2005; Sheng and Kim, 2002). Calcium signaling pathways also control neuronal differentiation, axon path finding, and dendrite morphogenesis (Wong and Ghosh, 2002).

A major player in all these processes is calcium/calmodulin-dependent protein kinase II (CaMKII), a calcium-activated serine/threonine kinase that is abundant in neurons, especially at postsynaptic sites (Lisman et al., 2002; Wong and Ghosh, 2002). The CaMKII holoenzyme, consisting of approximately 12 α and/or β subunits, is the most abundant constituent of the postsynaptic density (PSD) (Cheng et al., 2006). In mature hippocampal neurons, both CaMKIIα and CaMKIIβ are present at postsynaptic sites but seem to play different roles (Fink et al., 2003; Thiagarajan et al., 2002). Ca^{2+}/calmodulin binding to CaMKII subunits stimulates intersubunit Thr286 autophosphorylation (resulting in an activated kinase) and leads to phosphorylation of many substrates (Lisman et al., 2002).

Several CaMKII substrates, such as the NR2B subunit of NMDA receptors and the *Drosophila* homolog of mammalian CASK, Camguk, interact directly with CaMKII (Colbran and Brown, 2004; Griffith, 2004). These interactions differ in their dependence on Ca^{2+}/calmodulin binding and autophosphorylation and require different domains of the kinase; some even appear to be specific for either CaMKIIα (densin-180) or CaMKIIβ (F-actin) (Colbran and Brown, 2004). The variety of CaMKII interactions with neuronal proteins could target CaMKII to specific subcellular domains and modulate CaMKII activity during synapse formation (Fink et al., 2003), synaptic plasticity (Thiagarajan et al., 2002), axonal arborization, and dendrite morphogenesis (Gaudilliere et al., 2004; Wu and Cline, 1998). By functioning as a local calcium sensor, CaMKII exerts a critical influence on the architecture of the developing and plastic brain.

The liprinα family of proteins (liprinα1, -α2, -α3, -α4) was originally identified by its interaction with the leukocyte common antigen-related (LAR) family of receptor protein tyrosine phosphatases (LAR-RPTPs) (Pulido et al., 1995). Liprinα proteins consist of an N-terminal coiled-coil region that mediates homo- and heteromultimerization, followed by three SAM domains making up the liprin homology region (LH) that binds to the D2 (inactive phosphatase)

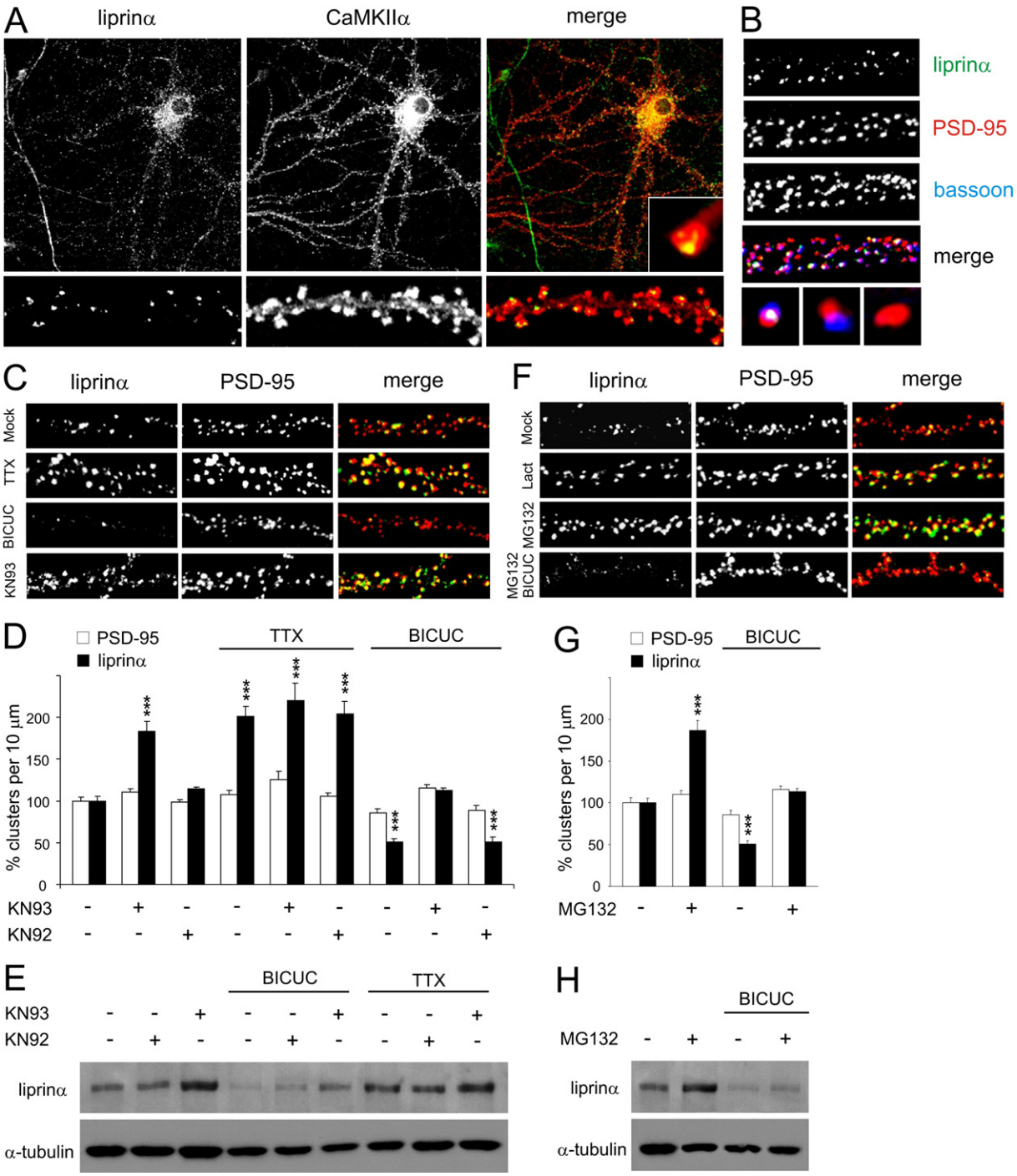


Figure 1. Regulation of Liprinα Levels by CaMKII and Proteasome in Hippocampal Neurons

(A) Representative images of rat hippocampal neurons (DIV13) double-labeled with rabbit anti-liprinα antibody (green) and mouse anti-CaMKIIα antibody (red). Only the merge is shown in color. Dendritic segments (lower panels) are enlarged to show colocalization of liprinα and CaMKIIα in spines. One single spine is enlarged (inset).

(B) Dendrites of hippocampal neurons triple-labeled with rabbit anti-liprinα antibody (green), guinea pig anti-PSD-95 antibody (red), and mouse anti-bassoon (blue). High-magnification panels (bottom) show three examples of synaptic clusters triple-stained for liprinα (green), PSD-95 (red), and bassoon (blue).

(C) Dendrites of hippocampal neurons at DIV17 treated with control vehicle (Mock), KN93 (10 μM), TTX (2 μM), or bicuculline (BICUC, 40 μM) for 24 hr and double-stained for liprinα (green) and PSD-95 (red), as indicated.

(D) Quantification of number of liprinα and PSD-95 clusters per 10 μm dendrite, normalized to control. Hippocampal neurons at DIV17 were treated for 24 hr with control vehicle (−) or KN92 or KN93 (+) in combination with TTX and bicuculline (BICUC), as indicated. Histograms indicate mean ± SEM.

domain of LAR-RPTPs (Serra-Pages et al., 1998). In cultured cell lines, liprinαs regulate LAR localization and clustering (Serra-Pages et al., 1995, 1998). In *Drosophila*, both liprinα and LAR are required for photoreceptor axon targeting in the visual system (Choe et al., 2006; Hofmeyer et al., 2006) and normal synaptic morphology at the larval neuromuscular junction (Kaufmann et al., 2002). Mutants in the *Caenorhabditis elegans* liprinα homolog *syd-2* display an abnormally diffuse distribution of presynaptic markers, lengthening of active zones, and impaired synaptic transmission (Zhen and Jin, 1999).

In mammals, liprinα proteins bind to several proteins present at presynaptic sites (Schoch and Gundelfinger, 2006). Together with data showing altered synaptic vesicle movement in *Drosophila* liprinα mutants (Miller et al., 2005), it is believed that in axons, liprinα has a role both in synaptic vesicle trafficking and active zone organization. However, liprinα is also localized in dendrites and postsynaptic sites, suggesting additional roles (Ko et al., 2003; Shin et al., 2003; Wyszynski et al., 2002). A postsynaptic function for liprinα has been described in hippocampal neurons, where liprinα binds to glutamate receptor interacting protein (GRIP) and regulates synaptic targeting of AMPA receptors (Wyszynski et al., 2002). In addition, liprinα associates with cadherin-β-catenin and, in conjunction with LAR-RPTPs, regulates its trafficking; in this way, liprinα is implicated in the development and/or maintenance of dendritic spines and excitatory synapses (Dunah et al., 2005).

Here we describe a novel activity-dependent regulation of liprinα by CaMKII, in which liprinα1 is degraded in response to CaMKII phosphorylation. Liprinα1 levels in hippocampal neurons are additionally regulated by the ubiquitin-proteasome system (UPS) via the E3 ubiquitin ligase anaphase promoting complex (APC). Expression of liprinα1 mutants insensitive to CaMKII degradation specifically inhibits the dendritic targeting of LAR receptors and leads to reduced dendrite arborization and synapse number. These findings provide a molecular basis for activity-dependent regulation of dendrite and synapse development by CaMKII, liprinα1, and LAR-RPTPs.

RESULTS

Downregulation of Liprinα in Hippocampal Neurons by CaMKII and Proteasome-Mediated Degradation

In screening for synaptic proteins whose abundance is regulated by synaptic activity, we discovered that liprinα levels fluctuate greatly in response to altered activity in cultured neurons. Liprinα proteins are present at both presynaptic and postsynaptic sites in the brain (Dunah et al., 2005; Wyszynski et al., 2002). In cultured hippocampal neurons (17 days in vitro; DIV17) fixed with ice-cold methanol, immunostaining with antibody made against liprinα1 shows a punctate pattern as previously described (Ko et al., 2003; Shin et al., 2003; Wyszynski et al., 2002), colocalizing with CaMKIIα in dendritic spines (Figure 1A). Liprinα puncta also showed extensive overlap with PSD-95, a postsynaptic density protein, and with bassoon, a presynaptic active zone protein (Figure 1B), indicating the presence of liprinα at synapses. The intensity of liprinα staining was generally weaker and more variable than the staining of these synaptic markers, such that some synapses had robust liprinα staining while other synapses showed undetectable signal (Figures 1A and 1B). Quantification revealed that ~90% of liprinα puncta overlapped with PSD-95 and bassoon clusters, but only ~30% of the PSD-95 and bassoon coclusters contained liprinα staining (Figure 1B; see Figure S1C in the Supplemental Data available with this article online).

Suppressing activity with tetrodotoxin (TTX, 2 μM, 24 hr) strongly increased liprinα expression, whereas increasing synaptic activity with the GABAA receptor antagonist bicuculline (40 μM, 24 hr) reduced liprinα expression in cultured hippocampal neurons (DIV17) (Figure 1C). The density of liprinα clusters doubled in TTX-treated cells, associated with increased immunofluorescence intensity of the clusters (Figures 1C and 1D; Figures S1A and S1C). Bicuculline profoundly reduced liprinα puncta density and brightness of staining. PSD-95 immunostaining showed similar bidirectional trends with altered activity, but the magnitude of fluctuation was much lower than that of liprinα (Figures 1C and 1D; Figures S1A and S1C). Western blotting confirmed that liprinα protein levels fell with increased activity (bicuculline) and rose with inactivity (TTX) (Figure 1E).

We tested whether CaMKII might play a role in the activity-dependent loss of liprinα, because this protein kinase is activated by synaptic excitation. Bath application of KN93, an inhibitor of CaMKII, strongly enhanced liprinα protein levels (Figure 1E) and increased the brightness and density of liprinα clusters (Figures 1C and 1D). KN92, an inactive analog of KN93, had no effect on liprinα levels by Western blot or by immunocytochemistry (Figures 1D and 1E). Again, PSD-95 showed a similar trend with KN93, but the degree of increase was much smaller than that of liprinα (Figures 1C and 1D). Thus, KN93 mimics the effect of TTX on liprinα expression. More importantly, KN93 prevented the effect of bicuculline and "rescued" liprinα back to control levels (Figures 1D and 1E); however, liprinα expression in neurons treated with both KN93 and bicuculline did not reach the high level

(E) Hippocampal cultures (DIV17) were treated for 24 hr with control vehicle (−) or KN92 or KN93 (+) in combination with TTX or BICUC, as indicated. Total lysates were immunoblotted for liprinα and α-tubulin as a loading control.
(F) Dendrites of hippocampal neurons at DIV17 treated with control vehicle (Mock), lactacystin (5 μM), MG132 (20 μM), or a combination of BICUC and MG132 for 24 hr and double-stained for liprinα (green) and PSD-95 (red).
(G) Number of liprinα and PSD-95 clusters per 10 μm dendrite (mean ± SEM). Hippocampal neurons at DIV17 were treated for 24 hr with control vehicle (−) or MG132 (+), with or without BICUC, as indicated. The number of clusters is normalized to unstimulated conditions.
(H) Hippocampal cultures (DIV17) were treated for 24 hr with control vehicle (−) or MG132 (+), with or without BICUC, as indicated. Total lysates were immunoblotted for liprinα and α-tubulin as a loading control. *$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$.

seen with KN93 alone (Figures 1D and 1E). These data indicate that CaMKII plays an important role in activity-dependent loss of liprinα, but suggest that additional independent mechanisms might be involved.

Because the UPS plays an important role in synaptic protein turnover (Ehlers, 2003), we tested whether proteasome inhibitors affected liprinα expression in hippocampal neurons. MG132 (20 μM, 24 hr) or lactacystin (5 μM, 24 hr) caused a robust increase in liprinα protein levels and in liprinα cluster density (Figures 1F–1H; Figures S1B and S1C). MG132 also blocked the reduction of liprinα by bicuculline (Figures 1F and 1G; Figures S1B and S1C); however, liprinα level or cluster number did not reach the high values seen with MG132 alone (Figures 1F–1H). These data indicate that proteasome-mediated degradation keeps the "basal" level of liprinα low and contributes substantially to activity-induced loss of liprinα; however, additional mechanisms seem to be involved, as noted above for CaMKII. Another possibility is that CaMKII- and proteasome-mediated downregulation of liprinα1 activity only partially depend on synaptic activity, as induced by bicuculline. Together, our results suggest that downregulation of liprinα in hippocampal neurons is mediated by CaMKII activity as well as proteasome degradation.

RNAi Knockdown of APC Increases Liprinα

In *Drosophila* neurons, liprinα may be regulated by APC, which acts as an E3 ubiquitin ligase (van Roessel et al., 2004). APC consists of >11 core subunits, including catalytic subunits APC2 and APC11, and is activated by regulatory subunits such as Cdh1 (Konishi et al., 2004). We tested whether APC might regulate liprinα1 in mammalian neurons by using small hairpin RNA (shRNA) expressed from the pSuper vector to knock down endogenous APC2 and Cdh1. Hippocampal neurons were transfected at DIV13 for 4 days with two independent APC2-shRNA (APC2-shRNA1 or APC2-shRNA2) or Cdh1-shRNA constructs, together with β-galactosidase (β-gal) to mark transfected neurons. To better preserve the β-gal staining, these cultures were fixed with formaldehyde, which results in a more diffuse staining of liprinα in dendrites in addition to the punctate synaptic pattern seen predominantly under methanol fixation conditions (Figures S2A and S2D). In neurons transfected with APC2-shRNA or Cdh1-shRNA constructs (Konishi et al., 2004), the intensity of liprinα immunostaining in dendrites was drastically increased (~100%–150% increase compared to control neurons) (Figures S2A and S2B). The intensity of PSD-95, revealed by double-staining, was unchanged in the same neuron (Figure S2C). Together with the MG132 and lactacystin results, these findings indicate that the UPS, particularly that mediated by APC, downregulates expression of liprinα in hippocampal neurons.

CaMKIIα/β Knockdown by RNAi Increases Liprinα in Hippocampal Neurons

To test which CaMKII isoform regulates liprinα levels in hippocampal neurons, we knocked down expression of endogenous CaMKIIα or CaMKIIβ by transfection of shRNA-expressing constructs. When tested in COS-7 cells, the CaMKIIα-shRNA construct specifically inhibited protein expression of CaMKIIα, and CaMKIIβ-shRNA specifically suppressed CaMKIIβ (Figure S3E). These RNAi constructs were cotransfected in hippocampal neurons (DIV13) with green fluorescent protein (GFP) to visualize morphology. Cells transfected with CaMKIIα-shRNA showed ~70% reduction in immunostaining for CaMKIIα in dendrites and cell body, with no change in CaMKIIβ staining intensity (Figures S3A–S3D). CaMKIIβ-shRNA-transfected neurons showed diminished staining for both CaMKIIβ (~80% reduction) and CaMKIIα (~60% reduction) (Figures S3A–S3D). Because CaMKIIβ-shRNA did not affect CaMKIIα expression in COS-7 cells, we believe that the loss of neuronal CaMKIIα induced by CaMKIIβ-shRNA is likely a secondary consequence of CaMKIIβ knockdown than due to nonspecificity of CaMKIIβ-shRNA (for instance, CaMKIIβ might be required for formation of stable CaMKIIα/β hetero-oligomeric holoenzymes).

We measured liprinα by immunostaining in hippocampal neurons transfected at DIV13 with CaMKIIα-shRNA, CaMKIIβ-shRNA, or both. β-gal was cotransfected to mark and outline the transfected cell. These cultures were fixed with formaldehyde. Four days after transfection of CaMKII-shRNA (DIV13+4), the integrated intensity per area (diffuse plus punctate staining) of liprinα immunostaining in the dendrite shaft was increased compared to control (~30% increase for CaMKIIα-shRNA, ~50% increase for CaMKIIβ-shRNA or CaMKIIα- + CaMKIIβ-shRNA) (Figures S2D and S2E). The intensity of PSD-95 staining was unchanged (data not shown). These RNAi data extend the KN93 pharmacological results, confirming that CaMKII inhibits liprinα protein expression in hippocampal neurons. At least the CaMKIIα isoform is involved; however, because CaMKIIβ-shRNA reduces expression of both CaMKIIα and CaMKIIβ, we cannot be certain whether CaMKIIβ directly regulates liprinα levels.

Active CaMKII Decreases Liprinα1 Protein Level in COS Cells

To investigate the mechanism by which liprinα1 expression is suppressed by CaMKII, we first turned to heterologous cells. In COS-7 cells, exogenous myc-tagged liprinα1 was expressed as a band of ~160 kDa (Figure 2A). Remarkably, when constitutively active CaMKIIα (T286D) was cotransfected, myc-liprinα1 levels became virtually undetectable (Figure 2A). Cotransfection of wild-type CaMKIIα(WT) or kinase-dead CaMKIIα(K42R) mutant did not affect liprinα1 protein levels (Figure 2A). A similar degree of suppression by cotransfected CaMKIIα(T286D) was seen for untagged liprinα1, liprinα1 tagged with GFP or HA, and liprinα1 expressed from different promoters (CMV, SV40, chicken β-actin), with or without the 3′ and 5′ untranslated regions of liprinα1 (data not shown). These results argue that CaMKII is not acting on the transcription or mRNA stability of liprinα1.

We also analyzed the effect of CaMKII on liprinα1 by an immunocytochemical assay. Liprinα1 cotransfected into

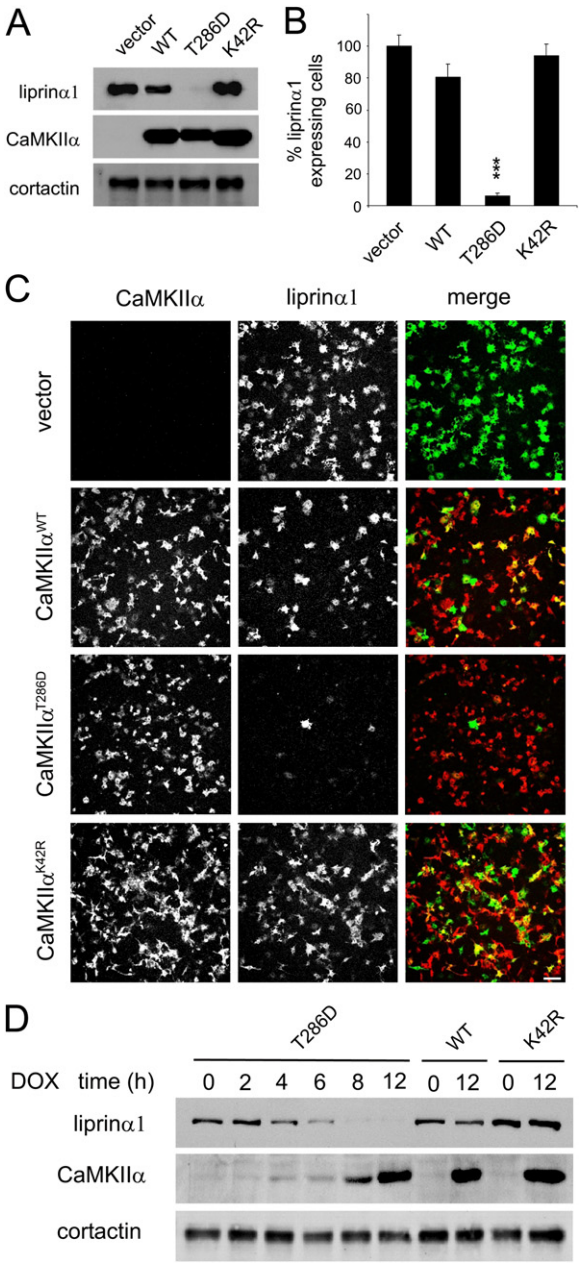


Figure 2. Induction of Active CaMKIIα Suppresses Liprinα1 Protein Levels

(A) COS-7 cells were double-transfected for 2 days with myc-liprinα1 plus empty vector control, wild-type (WT) CaMKIIα, constitutively active CaMKIIα(T286D), or kinase-dead CaMKIIα(K42R), as indicated. Whole-cell lysates from transfected COS-7 cells were immunoblotted for liprinα1 and CaMKII to detect the transfected constructs, and for endogenous cortactin as a loading control.

(B) Number of liprinα1-immunopositive cells (mean ± SEM; normalized to control) in COS-7 cultures transfected with GFP-liprinα1 and CaMKII constructs as in (C).

(C) Representative images of COS-7 cells cotransfected with GFP-liprinα1 (green) plus control vector, CaMKIIα(WT), CaMKIIα(T286D), or CaMKIIα(K42R) (red), as indicated. The merge is shown in color at right. The scale bar represents 100 μm. *p < 0.05, **p < 0.005, ***p < 0.0005.

(D) COS-7 cells were cotransfected with myc-liprinα1, pTRE-CaMKIIα (WT, T286D, or K42R), and rtTA expression construct. Two days after transfection, cells were treated with doxycycline (DOX) for various times as indicated (0–12 hr), and were immunoblotted for liprinα1 and CaMKII to detect the transfected constructs and for endogenous cortactin as a loading control.

COS-7 cells with empty vector control showed on average ~94 liprinα1-immunoreactive cells per 1.7 mm^2 in a 50%–70% confluent cell layer (Figures 2B and 2C). When liprinα1 was cotransfected with constitutively active CaMKIIα(T286D), however, only very few cells (~6 per 1.7 mm^2) expressed liprinα1 by immunostaining (Figures 2B and 2C). Cotransfection with wild-type (WT) or kinase-dead (K42R) CaMKIIα did not reduce the number of liprinα1-immunoreactive cells (Figures 2B and 2C).

To confirm that the loss of liprinα1 protein is due to the expression of CaMKIIα protein, we used a doxycycline (DOX)-inducible expression system (TETon) and followed over time the level of liprinα1 protein following the induction of active CaMKIIα. COS-7 cells were triply transfected with GFP-liprinα1, CaMKIIα(T286D) under the control of a tetracycline-responsive element (pTRE-CaMKIIα[T286D]), and a tetracycline transcriptional activator (rtTA) expression construct. At time 0 (no DOX added), liprinα1 protein was robustly detected by immunoblot in the absence of CaMKIIα signal (Figure 2D). From 4 to 12 hr after adding DOX, liprinα1 expression declined steadily, inversely correlated with a progressively rising CaMKIIα (Figure 2D). At 12 hr after DOX addition, liprinα1 was almost undetectable. No effect on liprinα1 protein expression was seen 12 hr after induction of CaMKIIα(K42R) or CaMKIIα(WT) using the same TETon system (Figure 2D). Similar results were obtained with these constructs using an immunocytochemical assay (Figures S4A–S4C). Induction of CaMKIIα(T286D), but not CaMKIIα(K42R) or CaMKIIα(WT), caused a dramatic reduction in the number of liprinα1-positive cells over time (Figures S4A–S4C; data not shown). These data provide compelling evidence that active CaMKIIα suppresses liprinα1 protein levels, with a time course suggesting active degradation of liprinα1 stimulated by CaMKIIα.

In COS-7 cells triply transfected with liprinα1, PSD-95, and CaMKIIα, PSD-95 protein level was unaffected by CaMKIIα(T286D), even while liprinα1 disappeared (Figure S4D). Immunocytochemistry analysis showed similar results: the percentage of PSD-95-immunoreactive COS cells was unaltered by cotransfection with CaMKIIα(T286D) (Figure S4E). CaMKIIα(T286D) also had no effect on GRIP1 (a multi-PDZ protein that binds to liprinα1 and AMPA receptors), cortactin, liprinα2, or liprinβ1 (proteins closely related to liprinα1) (Figures S4F and S4H). Thus, the effect of CaMKIIα(T286D) appears relatively specific for liprinα1.

Liprinα1 protein expression in COS cells was also strongly inhibited by cotransfection of active CaMKIIβ(T286D), but not by wild-type or inactive CaMKIIβ(K43R) (Figure S4G). Polo-like kinase-2 (Plk-2, also known as serum-inducible kinase; SNK) did not affect liprinα1 levels in COS cells

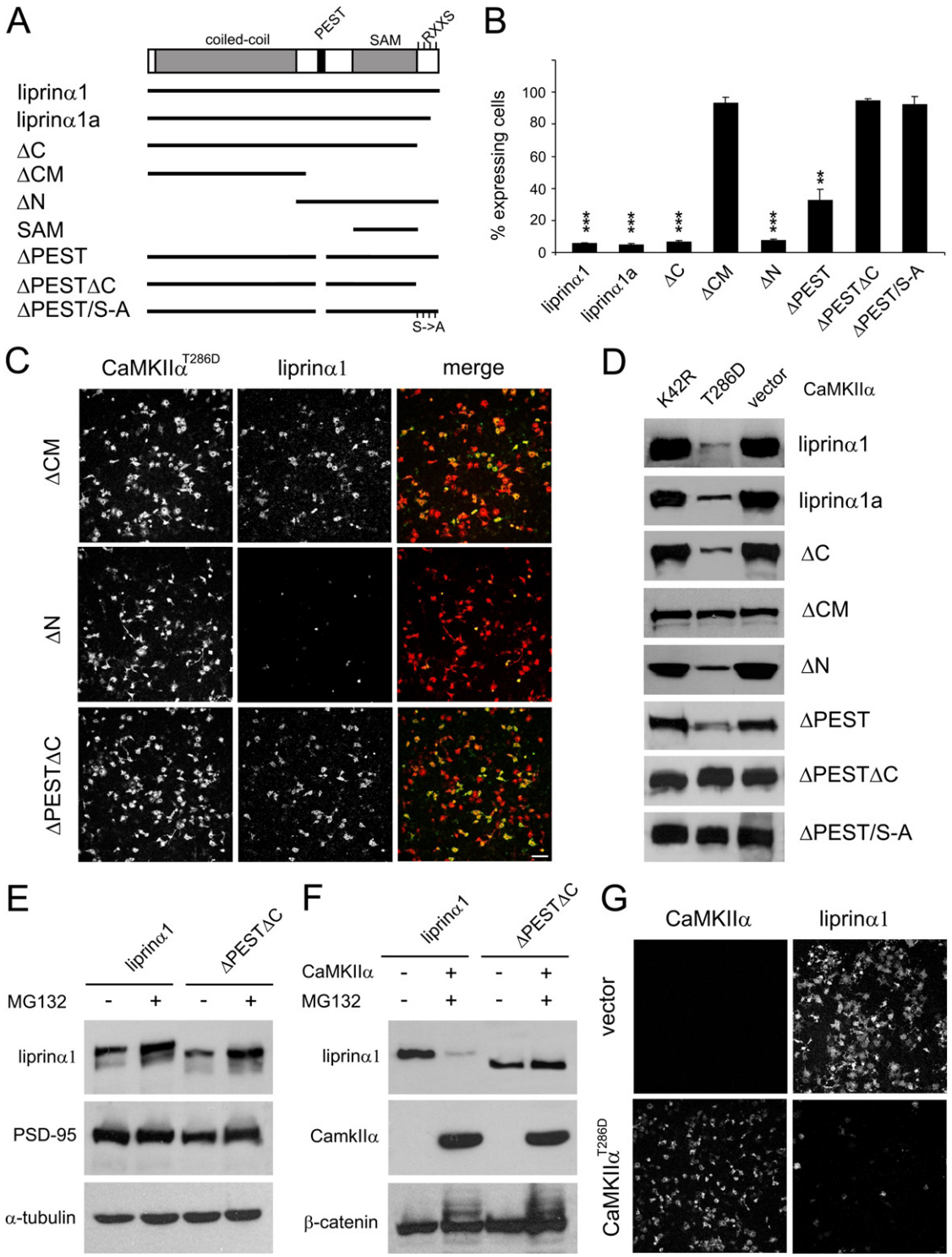


Figure 3. C Terminus and PEST Motif but Not Proteasome Are Required for CaMKII-Mediated Liprinα1 Degradation in COS-7 Cells

(A) Diagram of liprinα1 mutant constructs (GFP or HA tag was placed at N terminus).

(B) Number of cells immunopositive for indicated liprinα1 mutant constructs (mean ± SEM; normalized to control vector; not shown) when cotransfected with CaMKIIα(T286D). *$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$.

(C) Representative images of COS-7 cells cotransfected with CaMKIIα(T286D) (red) and GFP-liprinα1 mutant constructs (green), as indicated. Color merge is shown at right. The scale bar represents 100 μm.

(D) COS-7 cells cotransfected with indicated GFP-liprinα1 mutant constructs plus control vector, CaMKIIα(T286D), or CaMKIIα(K42R), and immunoblotted for the transfected liprinα1 construct using HA or GFP antibodies.

(data not shown), even though it strongly suppressed expression of SPAR, a Rap guanosine triphosphatase activating protein (Pak and Sheng, 2003). Thus, both CaMKIIα and CaMKIIβ can specifically suppress liprinα1 expression in heterologous cells, consistent with RNAi results obtained in neurons (see Figure S2). The data are most simply explained by active CaMKII stimulating the degradation of liprinα1.

C Terminus and PEST Motif Are Essential for CaMKII-Dependent Liprinα1 Degradation

As a first step toward the molecular mechanism behind this effect, we investigated the domains of liprinα1 required for CaMKII-mediated suppression (Figure 3A). GFP-tagged wild-type and deletion mutants of liprinα1 were cotransfected with CaMKIIα(T286D), CaMKIIα(K42R), or empty vector in COS-7 cells. Expression of liprinα1 constructs was quantified by immunocytochemistry and western blotting assays (Figures 3B–3D). The C-terminal region of liprinα1 contains four potential CaMKII phosphorylation sites (RXXS/T) which are conserved in human, rat, mouse, and chicken liprinα1 proteins. The liprinα1 splice variant lacking the last 18 amino acids (liprinα1a) (Wyszynski et al., 2002), which deletes two of the putative phosphorylation sites, and a C-terminal deletion mutant lacking the last 88 residues (liprinα1ΔC [1–1112]), which is missing all four phosphorylation sites, were still efficiently suppressed by CaMKIIα(T286D) and unaffected by CaMKIIα(K42R) (Figure 3D).

A mutant with a larger C-terminal truncation, liprinα1ΔCM (containing residues 1–712), was not degraded by CaMKIIα(T286D), implying that the C-terminal half of liprinα1 contains determinants for CaMKII-induced degradation (Figures 3B–3D). In this region there is a PEST (proline-, glutamate-, serine-, threonine-rich) sequence (amino acids 771–795), which is conserved among mammalian liprinα1 proteins, and which has a PEST score of +8.83 based on the PEST-FIND program (Rechsteiner and Rogers, 1996). A PEST score of more than 5 denotes a very strong proteolytic degradation signal. Deleting the PEST sequence (liprinα1ΔPEST) resulted in somewhat reduced susceptibility of liprinα1 to CaMKII suppression, compared with wild-type or ΔN (Figures 3B–3D). Combining the PEST deletion with the C-terminal deletion left a liprinα1 mutant (liprinα1ΔPESTΔC) that was completely insensitive to suppression by CaMKIIα(T286D) (Figures 3B–3D). We also mutated the serine residues of the putative C-terminal CaMKIIα phosphorylation sites to alanine (S1139A, S1168A, S1194A, S1201A) in a ΔPEST background, giving rise to liprinα1ΔPEST/S-A. The liprinα1ΔPEST/S-A mutant also was insensitive to CaMKIIα(T286D) (Figures 3B and 3D). Thus, both the central PEST sequence and the C-terminal putative CaMKII phosphorylation sites are important for liprinα1 degradation by active CaMKII.

Proteasome Is Not Involved in CaMKIIα-Dependent Liprinα1 Degradation in COS-7 Cells

The PEST motif is known to promote rapid protein turnover; however, the pathways responsible for degrading PEST proteins are not always clear. Several PEST proteins have been reported to be degraded by calpain or proteasome, but degradation of other PEST-containing proteins seems independent of these mechanisms (Rechsteiner and Rogers, 1996). Two distinct proteasome inhibitors, MG132 and lactacystin, increased the basal level of both liprinα1 and liprinα1ΔPESTΔC expressed in COS-7 cells (Figure 3E; data not shown), implying that the proteasome is involved in downregulating basal liprinα1 levels. In contrast, expression of cotransfected PSD-95 was unaffected (Figure 3E). Importantly, however, 4, 8, and 12 hr MG132 treatment did not prevent the loss of liprinα1 induced by cotransfection of CaMKIIα(T286D) (Figures 3F and 3G; data not shown), indicating that proteasome function is not required for elimination of liprinα1 by active CaMKII. In addition, liprinα1 degradation by active CaMKII was not blocked by calpain inhibitors (ALLN, ALLM) or inhibitors of lysosomal degradation (chloroquine, leupeptin, ammonium sulfate) (data not shown). Overall, these data indicate that although it contributes to basal lowering of liprinα1, proteasomal activity is not essential for CaMKII-mediated degradation of liprinα1 in heterologous cells.

Interaction of CaMKII and Liprinα1 In Vitro and In Vivo

Our mutational analysis suggests that liprinα1 is a target of CaMKII phosphorylation; therefore, we tested whether liprinα1 and CaMKII interact biochemically. From cotransfected COS-7 cells, liprinα1 was readily coimmunoprecipitated with CaMKIIα wild-type and K42R (Figures 4A and 4B). For this experiment, we could not use the CaMKIIα(T286D) construct, as active CaMKII causes a drastic reduction in the amount of liprinα1. Nonimmune rabbit IgG immunoprecipitated neither liprinα1 nor CaMKII, and liprinα2, cortactin, and PSD-95 could not be coprecipitated with wild-type CaMKIIα (Figures 4A–4C), indicating a specific interaction between liprinα1 and CaMKIIα. The association with CaMKIIα was lost in the liprinα1 mutant ΔCM but retained in ΔN, indicating that CaMKII interacts with the C-terminal half of liprinα1 (Figure 4D). Interestingly, the mutant ΔPESTΔC, although insensitive to CaMKII-mediated degradation, could be coimmunoprecipitated

(E) COS-7 cells cotransfected with myc-PSD-95 plus GFP-liprinα1 or GFP-liprinα1ΔPESTΔC were treated (+) or not treated (−) with 5 μg/ml MG132 for 4 hr and immunoblotted for myc and GFP. Endogenous α-tubulin was used as a loading control.

(F) COS-7 cells transfected with GFP-liprinα1 or GFP-liprinα1ΔPESTΔC plus control vector (−) or CaMKIIα(T286D) (+) were treated (+) or not treated (−) with 5 μg/ml MG132 for 4 hr as indicated, and immunoblotted for GFP or CaMKIIα. Endogenous β-catenin was used as a positive control for MG132 treatment.

(G) Representative images of COS-7 cells cotransfected with GFP-liprinα1 (green) and control vector or CaMKIIα(T286D) (red), and treated with 5 μg/ml MG132 for 4 hr.

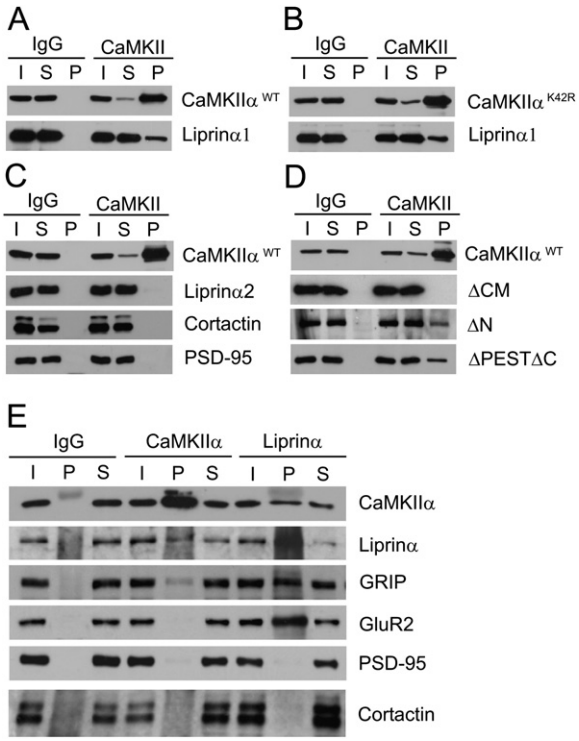


Figure 4. CaMKII Interaction with Liprinα1 In Vitro and In Vivo

(A and B) COS-7 cells cotransfected with wild-type (WT) GFP-CaMKIIα and myc-liprinα1 (A), or GFP-CaMKIIα(K42R) and myc-liprinα1 (B), were immunoprecipitated with nonimmune rabbit IgG or CaMKII antibodies. Each immunoprecipitation reaction is shown in three lanes: I, input to IP reaction; S, supernatant remaining after IP; P, precipitated pellet. The I, S, and P samples were immunoblotted for the indicated proteins.

(C and D) COS-7 cells cotransfected with GFP-CaMKIIα(WT) and indicated proteins (C) or liprinα1 mutant constructs (D) were immunoprecipitated and immunoblotted as in (A) and (B).

(E) Coimmunoprecipitation of CaMKII and liprinα from rat cortex. Deoxycholate extracts were immunoprecipitated with nonimmune rabbit IgG or CaMKIIα or liprinα antibodies, and immunoblotted for the proteins indicated at right.

with CaMKII (Figure 4D), implying that the SAM domains are responsible for interaction with CaMKII (see Figure 3A for diagram). Notable in this respect is the recent discovery that the CaMKII-like domain of scaffold protein CASK interacts with the SAM region of liprinα1 (Olsen et al., 2005).

To test for an in vivo interaction, we performed coimmunoprecipitations from deoxycholate extracts of rat cerebral cortex. Consistent with earlier studies (Dunah et al., 2005; Wyszynski et al., 2002), GRIP and GluR2/3 were robustly coprecipitated with the liprinα antibody (Figure 4E). Liprinα antibodies also precipitated significant amounts of CaMKIIα. In the inverse reaction, CaMKIIα antibodies brought down a small amount of liprinα and GRIP in addition to precipitating CaMKIIα, but no detectable GluR2/3 (Figure 4E). As a negative control, PSD-95 and cortactin were not coprecipitated by liprinα or CaMKIIα antibodies and none of the analyzed proteins was pelleted by nonimmune rabbit IgG (Figure 4E). These biochemical data suggest that a subset of CaMKIIα is present in some fraction of the liprinα protein complex in vivo as well as in vitro, which is consistent with the colocalization of liprinα and CaMKII clusters at synaptic sites (Figures 1A and 1B).

Liprinα1ΔPESTΔC and Liprinα1ΔPEST/S-A Inhibit Dendrite Morphogenesis and Reduce Synapse Density

What is the functional significance in neurons of liprinα1 degradation by CaMKII? To address this question, we transfected hippocampal neurons (DIV13) with liprinα1 wild-type versus liprinα1ΔPESTΔC or liprinα1ΔPEST/S-A mutants that can no longer be degraded by CaMKII. Note, however, that these mutants are sensitive to proteasome downregulation, insofar as their basal levels are increased by MG132 (Figure 3E). Neuronal morphology was visualized by cotransfected monomeric red fluorescent protein (mRFP). Four days after transfection, neurons transfected with liprinα1ΔPESTΔC or liprinα1ΔPEST/S-A showed stunted dendritic arbors relative to neurons transfected with liprinα1 (Figure 5A; data not shown). Compared with vector control, overexpression of wild-type liprinα1 had no effect on any dendrite parameter measured (total dendritic length, number of primary dendrites or dendrite tips, and density or size of dendritic protrusions) (Figures 5A and 5D–5F). Total dendritic length decreased by ~30% in liprinα1ΔPESTΔC- and liprinα1ΔPEST/S-A-transfected neurons (Figures 5A and 5D). Dendrite branching was also reduced, as quantified by total number of dendrite tips (~40% decrease; Figure 5E) or by Sholl analysis (which measures the number of dendrites crossing circles at various radial distances from the cell soma; Figure 5C). Liprinα1ΔPESTΔC and liprinα1ΔPEST/S-A also decreased (by ~25%) the number of primary dendrites (defined as dendrites longer than 21 μm emanating directly from the soma) (Figure 5F). Although overexpression of wild-type liprinα1 had no effect on dendrite morphology, KN93 treatment of wild-type liprinα1-transfected cells mimicked the effects of overexpression of CaMKII-nondegradable mutants on dendrite morphology (Figures 5A and 5C–5F). As expected, levels of wild-type liprinα1 protein were increased in cells treated with KN93. In summary, the expression of "stable" liprinα1 caused a decrease in the number of primary, secondary, and higher-order dendrites and a reduction in the dendritic arbor complexity in cultured hippocampal neurons. We conclude that CaMKII-mediated degradation of liprinα1 is essential for normal growth and maturation of the dendritic tree.

The density of dendritic protrusions and spines (defined as protrusions of 1–4 μm in length that showed a clear "head") at DIV17 was reduced by liprinα1ΔPESTΔC and liprinα1ΔPEST/S-A, but unaffected by wild-type liprinα1 (Figures S5A and S5B). The mean length and width of remaining spines were not substantially different in neurons overexpressing any of the liprinα1 constructs (Figures S5A, S5C, and S5D). In accord with a reduction in the number of spines, we observed an ~40% fall in the density of bassoon puncta on neurons transfected with

liprinα1ΔPEST/S-A (liprinα1: 4.89 ± 0.21 per 10 μm length of dendrite versus liprinα1ΔPEST/S-A: 2.74 ± 0.28 per 10 μm length of dendrite), an indication that neurons expressing CaMKII-insensitive liprinα1 harbor fewer presynaptic contacts. Thus, in addition to reduced dendrite branching, CaMKII-nondegradable liprinα1 mutants impair the development and/or maintenance of spines and synapses.

LAR-Liprin Interfering Constructs and LAR-shRNA Inhibit Dendrite Morphogenesis

We investigated which domain of liprinα1 is important for dendrite morphology by overexpressing liprinα1 deletion constructs liprinα1ΔN, liprinα1SAM, and liprinα1ΔCM (see Figure 3A), assuming that the mutants work as dominant negatives. Four days after transfection, neurons expressing liprinα1ΔN and liprinα1SAM showed shorter dendritic arbors relative to neurons transfected with control vector or liprinα1ΔCM (Figures 5B and 5D), similar to liprinα1ΔPESTΔC- and liprinα1ΔPEST/S-A-transfected cells. Dendrite branching was also reduced, as quantified by dendrite tips (~30% decrease; Figure 5E), primary dendrites (~25% decrease; Figure 5F), and Sholl analysis (Figure 5C). The density of dendritic protrusions and spines at DIV17 was reduced by liprinα1ΔN and liprinα1-SAM, but unaffected by liprinα1ΔCM (Figures S5A–S5D). These data argue that the SAM domain of liprinα1 is important for supporting dendrite morphogenesis and normal spine number.

The LAR-RPTPs bind to the SAM domains in liprinα1 (Serra-Pages et al., 1995). Therefore, we investigated the effect on dendrite morphogenesis of overexpression of the isolated liprinα binding domain of LAR (LAR-D2, fused to a myristoylation motif for membrane targeting), or knockdown of LAR by LAR-shRNA (Dunah et al., 2005). Expression of either LAR-D2 or LAR-shRNA caused marked pruning of the dendritic arbor (Figures 5B–5F) and loss of dendritic protrusions and spines (Figures S5A–S5D). In summary, the expression of constructs that disrupt the liprinα1-LAR interaction or that suppress endogenous LAR expression caused a reduction in dendrite arborization and spine density in hippocampal neurons, similar to the effects seen with neurons expressing CaMKII-nondegradable liprinα1 constructs.

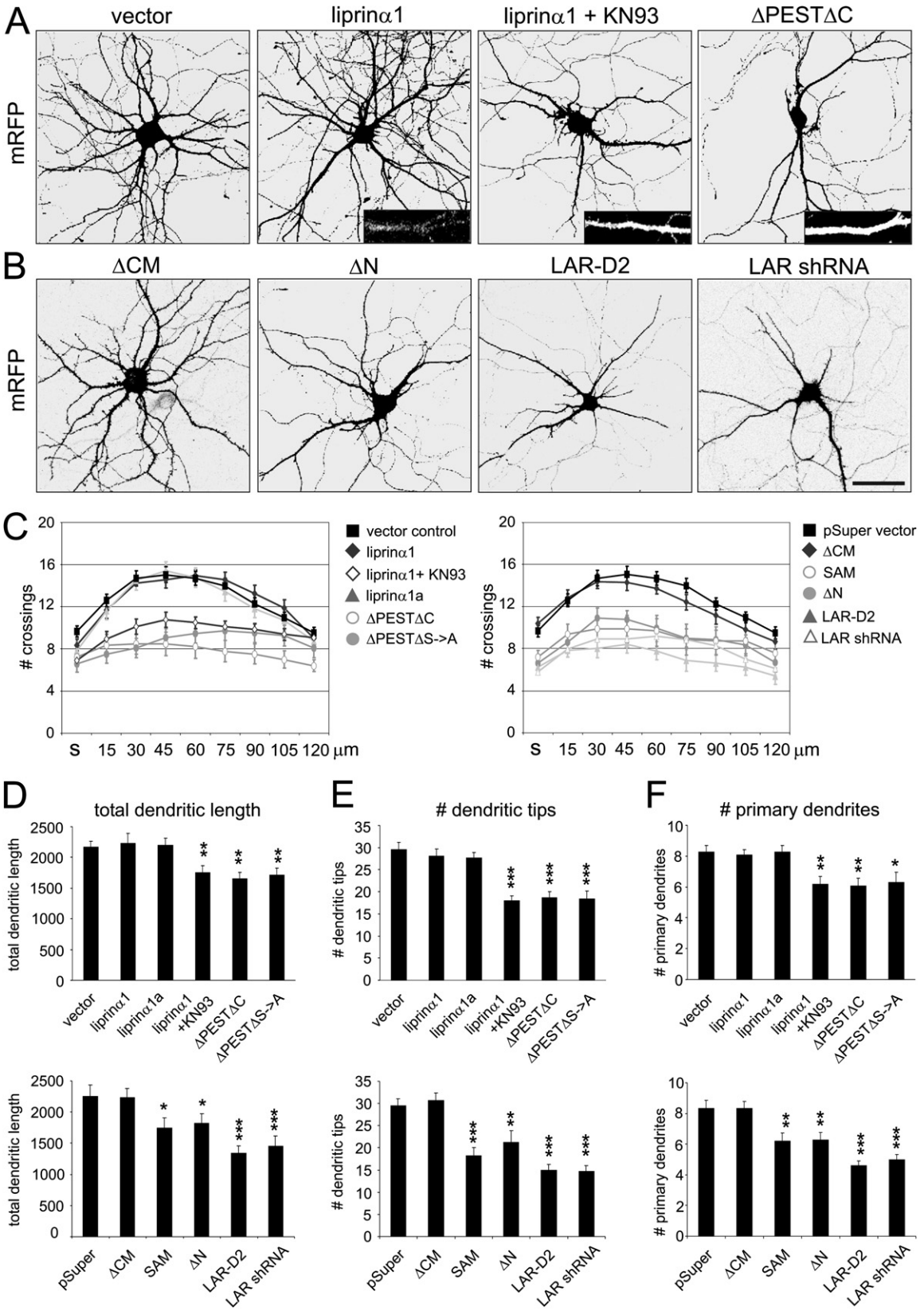
Liprinα1 Increases Surface Expression and Clustering of LAR Receptors

Liprinα has been suggested to regulate LAR localization and clustering in mammalian cell lines (Serra-Pages et al., 1995, 1998). It is difficult to study the regulation of endogenous LAR trafficking in neurons because LAR protein is not highly expressed and our LAR antibodies work insufficiently in immunostaining. We developed a simplified way to investigate LAR receptor trafficking, which makes use of the CD8 glycoprotein as a reporter in protein trafficking (Hoogenraad et al., 2005). A GFP-tagged CD8 construct containing only the extracellular and transmembrane domain of CD8 accumulated in the endoplasmic reticulum of HeLa cells, colocalizing with calreticulin (Figures S6A and S6B); expression on the cell surface was weak (Figure S6C). Fusing the entire cytoplasmic domain of LAR (LARC) to the CD8 construct allowed the chimeric construct (CD8-LARC) to leave the endoplasmic reticulum and accumulate in the perinuclear Golgi region (Figure S6D), where it colocalized with the trans-Golgi marker BICD2 (Figure S6E).

In the absence of coexpressed liprinα1, very little CD8-LARC was associated with the cell surface (Figure 6E). Cotransfection with liprinα1, liprinα1ΔPESTΔC, or liprinα1ΔPEST/S-A resulted in greatly increased expression of CD8-LARC at the surface in a patchy pattern in almost all cells (Figures 6A, 6B, and 6F). Coexpression of liprinα1ΔCM, lacking the LAR binding domain, did not change the distribution of CD8-LARC, which remained in a Golgi pattern (Figure 6C). Similarly, the CD8-LARCΔD2 construct lacking the liprin binding site was unaffected by cotransfection of liprinα1ΔPESTΔC (Figure 6D). These data in heterologous cells suggest that one function of liprinα1 (both wild-type and CaMKII-nondegradable mutants) is to drive LAR receptors out of the Golgi and deliver them to the cell surface.

CaMKII-Nondegradable Liprinα1 Mutants Impair Dendritic Targeting of LAR

We next investigated LAR trafficking in hippocampal neurons. When overexpressed in neurons, CD8-LARC was found, in addition to the perinuclear Golgi region (Figure S7B), in a punctate pattern in dendrites (Figure 7A, left, inset, arrowheads) and at low levels on the cell surface (Figure S7A). Overexpression of wild-type liprinα1 has no effect on the CD8-LARC distribution (Figure 7B). However, cotransfection of liprinα1ΔPESTΔC and liprinα1ΔPEST/S-A mutants caused redistribution of CD8-LARC from Golgi and accumulation of CD8-LARC in neuronal cell bodies (Figures 7C–7E; Figure S7B). In these neurons, CD8-LARC was predominantly found in a patchy pattern along the periphery of the cell body, suggesting an increased surface expression on the soma (Figure 7C; Figure S7B). The ratio of cell body (soma without the Golgi)/Golgi immunostaining for CD8-LARC increased 2-fold in liprinα1ΔPESTΔC- and liprinα1ΔPEST/S-A-expressing neurons compared to control (Figure 7E), indicating improved exit from Golgi toward the somatic surface, similar to the findings in HeLa cells. Despite the increased expression on neuronal somata, the punctate staining of CD8-LARC in the dendrites was greatly reduced (Figure 7C). The ratio of dendrite/Golgi immunostaining for CD8-LARC decreased 2-fold in neurons expressing liprinα1ΔPESTΔC or liprinα1ΔPEST/S-A (Figure 7D), signifying that CaMKII-nondegradable liprinα1 mutants impair dendritic targeting of LAR, despite improving Golgi-to-somatic surface transport. The distribution of CD8-LARCΔD2, lacking the liprinα binding site, was unaffected by CaMKII-nondegradable liprinα1 constructs (Figures 7D and 7E). In neurons transfected with HA-liprinα1 and treated with KN93 (10 μM, 24 hr), the ratio of cell body/Golgi intensity of CD8-LARC immunostaining increased 2-fold and the dendrite/Golgi ratio decreased 2-fold compared to untreated


A
vector
liprinα1
liprinα1 + KN93
ΔPESTΔC
mRFP
B
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liprinα1-transfected cells (Figures 7D and 7E), mimicking the effect of overexpressed CaMKII-nondegradable liprinα1 mutants. Thus, prevention of liprinα1 degradation by CaMKII, either by mutation of liprinα1 or by drug inhibition of CaMKII, reduces the trafficking of CD8-LARC to dendrites. Together, these data indicate that liprinα1 degradation by CaMKII is needed specifically for the dendritic targeting of LAR, thereby promoting normal development of dendrites and synapses.

DISCUSSION

Activity-Dependent Regulation of Liprinα1 by Two Mechanisms: CaMKII and Proteasome

Activity regulates the expression and degradation of many neuronal proteins, including several scaffolds of the PSD (Ehlers, 2003). One of the major cellular mechanisms controlling protein turnover is the UPS (Hegde and DiAntonio, 2002; Murphey and Godenschwege, 2002). Ubiquitin-processing enzymes play an essential role in neural development, including synapse growth and development, growth cone guidance, and dendritic remodeling (Kuo et al., 2005; Murphey and Godenschwege, 2002). In most cases, the importance of the UPS for neural development is inferred from pharmacological inhibitor or genetic loss-of-function studies, and the specific proteins whose level is controlled by ubiquitination and proteasomal proteolysis are unknown. Here we found liprinα1 levels to be particularly susceptible to activity-dependent regulation. The suppression of liprinα1 protein by neural activity depends at least partially on CaMKII and proteasome function. Because inhibitors of either CaMKII or proteasome countered the effect of activity but did not elevate liprinα1 to the high levels seen with the inhibitors alone, we hypothesize that synaptic activity stimulates at least two pathways for liprinα1 degradation: one depending on CaMKII phosphorylation, and another depending on the UPS. This idea is supported by the fact that proteasome inhibitors do not prevent the degradation of liprinα1 by CaMKII in COS cells. In addition, the data in neurons can be explained if CaMKII- and proteasome-mediated turnover of liprinα1 depend only partially on synaptic activity. Cotreatment of neurons with KN93 and MG132 did not significantly increase liprinα1 levels compared to either drug alone (data not shown), which does not support the idea that CaMKII and proteasomes lie in independent pathways for liprinα1 degradation. Thus, our findings in heterologous cells do not rule out that CaMKII degradation of liprinα1 occurs via the UPS in neurons, or that crosstalk occurs between these pathways.

Our findings indicate that CaMKII affects liprinα1 levels via protein degradation as opposed to a reduction in transcription or translation. To our knowledge, this is the first example of CaMKII signaling in which CaMKII activity stimulates degradation of a specific protein. The degradation of liprinα1 by CaMKII involves a central PEST sequence and C-terminal phosphorylation sites in liprinα1. At least 12 CaMKII consensus phosphorylation sites are present in liprinα1. Phosphorylation of the C-terminal tail and CaMKII sites close to the PEST sequence may recruit degradation factors necessary for proteolysis (Garcia-Alai et al., 2006). Alternatively, CaMKII phosphorylation may induce conformation changes in liprinα1 which open up additional regions involved in rapid protein degradation. Although mutation analysis in liprinα1 suggests a direct role for CaMKII phosphorylation in liprinα1 degradation, we cannot rule out that another factor is activated by CaMKII which subsequently causes liprinα1 degradation. A PEST motif is absent in liprinα2, which is not degraded by active CaMKII, but present in the middle part of liprinα4, which additionally contains two CaMKII phosphorylation sites in its C terminus. Thus, liprinα4 might also be degraded by active CaMKII.

Given that liprinα1 exists on both sides of mammalian excitatory synapses (Dunah et al., 2005; Ko et al., 2003; Shin et al., 2003; Wyszynski et al., 2002), does activity regulate the level of liprinα at pre- or postsynaptic sites? CaMKII is extremely abundant in the postsynaptic density (Cheng et al., 2006; Lisman et al., 2002), but also exists in axon terminals (Liu and Jones, 1997). Moreover, activity results in Ca^{2+} elevation in both pre- and postsynaptic compartments. Light microscopy cannot definitely distinguish between pre- and postsynaptic accumulation of liprinα1. However, we believe that the regulation of liprinα1 levels by CaMKII occurs substantially in the postsynaptic compartment because RNAi knockdown of CaMKII in neurons resulted in increased immunostaining of dendritic liprinα1 in a cell-autonomous fashion (Figure S2). Regulation of liprinα1 by APC must also occur at least in part on the postsynaptic side, because Cdh1 RNAi boosted liprinα1 signal in dendrites of transfected neurons. Finally, CaMKII-insensitive liprinα1 caused robust changes in dendrite morphology in transfected neurons, implying that this mutant can have a postsynaptic (or dendritic) locus of action. Nevertheless, our study does not rule out an effect of CaMKII or APC on liprinα1 in presynaptic compartments.

Figure 5. Impairment of Dendrite Morphogenesis by Liprinα1 Mutants Insensitive to CaMKII Degradation, LAR-Liprin Interfering Constructs, and LAR-shRNA

(A) Morphology of hippocampal neurons (visualized in the RFP channel) cotransfected at DIV13 for 4 days with control vector, GFP-liprinα1, or GFP-liprinα1ΔPESTΔC, plus mRFP as transfection marker. "+ KN93" indicates treatment for 24 hr with 10 μM KN93 to inhibit CaMKII. GFP-liprinα1 signals are shown in insets.

(B) Representative images (RFP channel) of hippocampal neurons cotransfected at DIV13 for 4 days with control vector, LAR-liprin interfering constructs, or LAR-shRNA, plus mRFP to visualize the transfected cell. The scale bar represents 20 μm.

(C) Sholl analysis of hippocampal neurons transfected at DIV13 for 4 days with liprinα1, LAR, and shRNA constructs.

(D–F) Quantification of total dendritic length (D), number of dendritic tips (E), and number of primary dendrites (F) in hippocampal neurons transfected at DIV13 for 4 days with indicated constructs (mean ± SEM). *$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$.

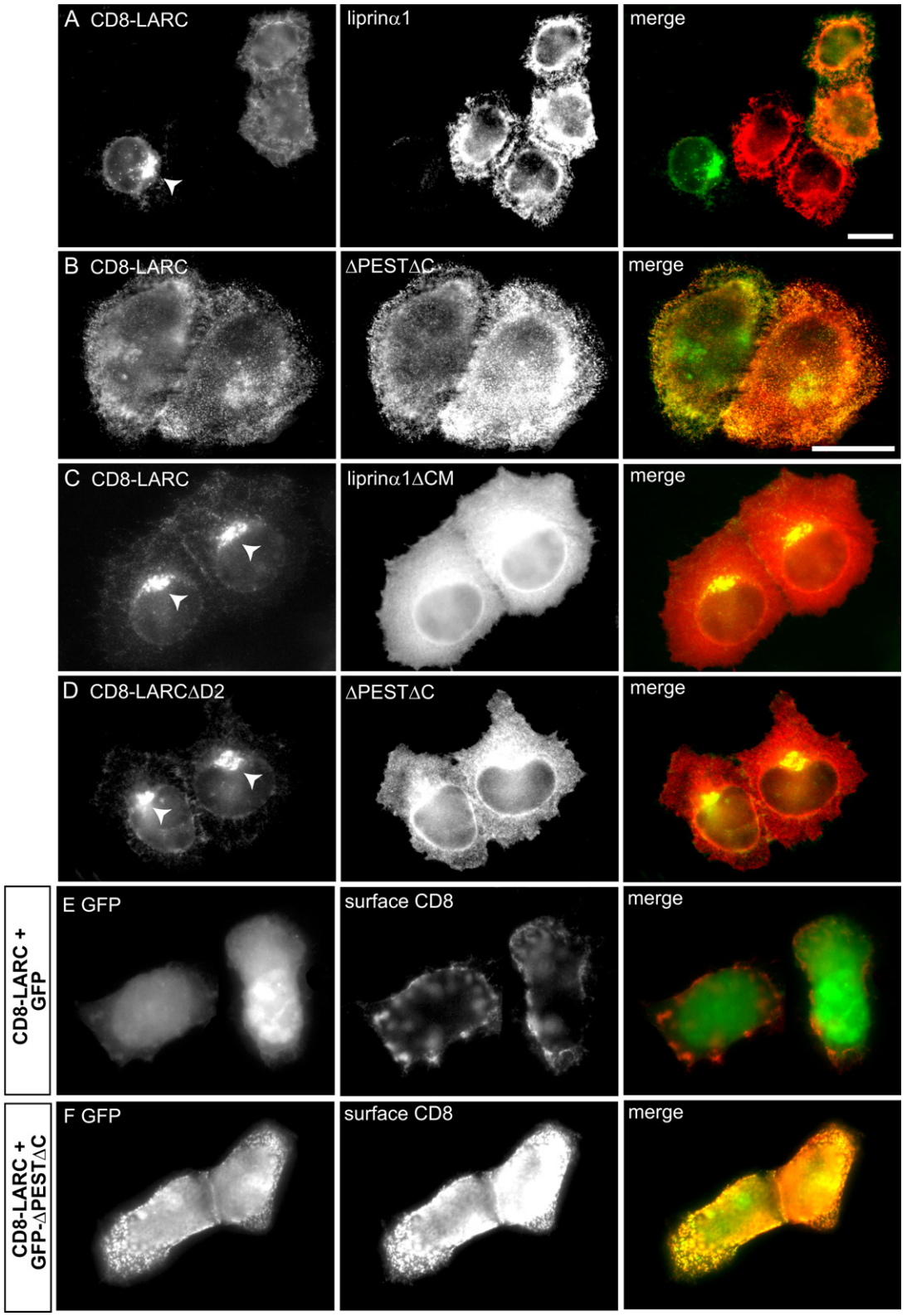


Figure 6. Liprinα1 and Liprinα1 Mutants Insensitive to CaMKII Degradation Promote CD8-LARC Exit from Golgi and Surface Expression in HeLa Cells

(A–D) HeLa cells were double-transfected with CD8-LARC plus HA-liprinα1 (A), HA-liprinα1ΔPESTΔC (B), HA-liprinα1ΔCM (C), or with CD8-LARCΔD2 plus HA-liprinα1ΔPESTΔC (D), and then fixed and stained with anti-HA (red) and anti-CD8 (green) antibodies. The merge is shown in color at right.

Importance of CaMKII Degradation of Liprinα1 for Dendrite Morphogenesis

CaMKII has been implicated in neuronal morphogenesis and synapse maturation (Lisman et al., 2002; Wong and Ghosh, 2002). CaMKII signaling can regulate dendrite development by local processes or by inducing transcriptional programs in the nucleus (Gaudilliere et al., 2004). CaMKII activity promotes growth and stabilization of dendrites in *Xenopus* optic tectal neurons (Wu and Cline, 1998) and of dendritic spines in hippocampal neurons (Fink et al., 2003). The precise mechanisms by which CaMKII influences dendrite and synapse development remain poorly understood. Based on our findings, we propose that CaMKII-mediated phosphorylation and degradation of liprinα1 is one molecular mechanism for coupling activity to dendrite and synapse morphogenesis. Liprinα1 mutants resistant to CaMKII degradation impair dendrite arborization and synapse density; thus, the ability of liprinα1 protein to be degraded in response to CaMKII activation is essential for normal dendrite and synapse development. Because CaMKII can be stimulated locally by synaptic activity, suppression of liprinα1 by CaMKII might be used to promote dendrite growth or stability locally in regions of high synaptic activity. Our data correlate well with previous studies showing that activity—specifically stimulation of glutamate receptors in mature neurons—stabilizes dendritic arbors (Cline, 2001), and that inhibition of CaMKII with RNAi or peptide inhibitors reduces dendritic arborization and synapse formation (Fink et al., 2003).

How does liprinα1 regulate dendrite morphology? Two obvious candidates are the known liprinα1 binding proteins GRIP1 (Wyszynski et al., 2002) and LAR-RPTP (Serra-Pages et al., 1998, 2005). GRIP1 has already been implicated in dendrite development (Hoogenraad et al., 2005); however, CaMKII-nondegradable mutants of liprinα1 had no effect on the distribution of GRIP1 in hippocampal neurons (data not shown). Here we show that RNAi knockdown of LAR and disruption of the LAR-liprinα interaction reduce dendritic arbor complexity in cultured hippocampal neurons very similarly to that seen with CaMKII-nondegradable liprinα1 mutants. Previous work has suggested that liprinα is involved in the localization and distribution of LAR in mammalian cells (Serra-Pages et al., 1995, 1998). Our experiments indicate that liprinα1 drives LAR receptors out of the Golgi and enhances LAR expression and clustering on the neuronal cell body, likely associated with an increased surface expression. This function was independent of liprinα1 degradation by CaMKII in that ΔPESTΔC and ΔPEST/S-A mutants of liprinα1 were fully active in this respect. However, specifically the targeting of LAR to dendrites was abrogated by liprinα1 mutants that are immune to CaMKII degradation, as well as by KN93 block of CaMKII activity, even though both manipulations greatly increase liprinα1 levels in neurons. Because postsynaptic LAR is critical for dendrite development and synapse/spine maturation (this study; Dunah et al., 2005), the loss of dendritic targeting of LAR can largely explain the phenotype of CaMKII-nondegradable liprinα1 overexpression.

So why is the *degradation* of liprinα1, rather than just its expression, important for LAR distribution and dendrite and synapse development? Although we can only manipulate CaMKII activity or liprinα1 expression at a global level within individual neurons, the physiological role of CaMKII regulation of liprinα-LAR occurs most likely at the local level. We hypothesize that the local regulation of liprinα1 levels by CaMKII controls the trafficking of LAR to specific regions within dendrites and/or to specific synapses. The CaMKII-mediated degradation of liprinα1 provides an attractive mechanism for targeting liprinα1-LAR complexes to active synapses where the kinase is switched "on." We suggest that at such sites, the LAR cargo bound to liprinα1 would be "unloaded" due to CaMKII-mediated degradation of liprinα1 to promote local growth and stabilization of dendritic structures. However, it is possible that the mutations that block CaMKII-mediated degradation interfere in some other way with liprinα1's trafficking function in dendrites.

In yeast, a specific myosin motor, Myo2p, moves vacuoles to the yeast bud by binding to the vacuole-specific Myo2p receptor Vac17p (Tang et al., 2003). The transport complex is disrupted specifically in the bud by degradation of Vac17p, depositing the vacuole in the bud. Vac17p contains a PEST sequence that is required for its degradation, and loss of this PEST sequence causes mistargeting of vacuoles (Tang et al., 2003). By analogy to Vac17p, liprinα1 also interacts with motor proteins (kinesin-1 and kinesin-3; KIF1A) (Miller et al., 2005; Shin et al., 2003). Liprinα1 also contains a PEST sequence important for rapid degradation, and causes a cargo targeting phenotype when it is rendered nondegradable. An attractive idea is that PEST protein degradation in yeast and hippocampal neurons represent different aspects of a general molecular mechanism for directed motor trafficking.

EXPERIMENTAL PROCEDURES

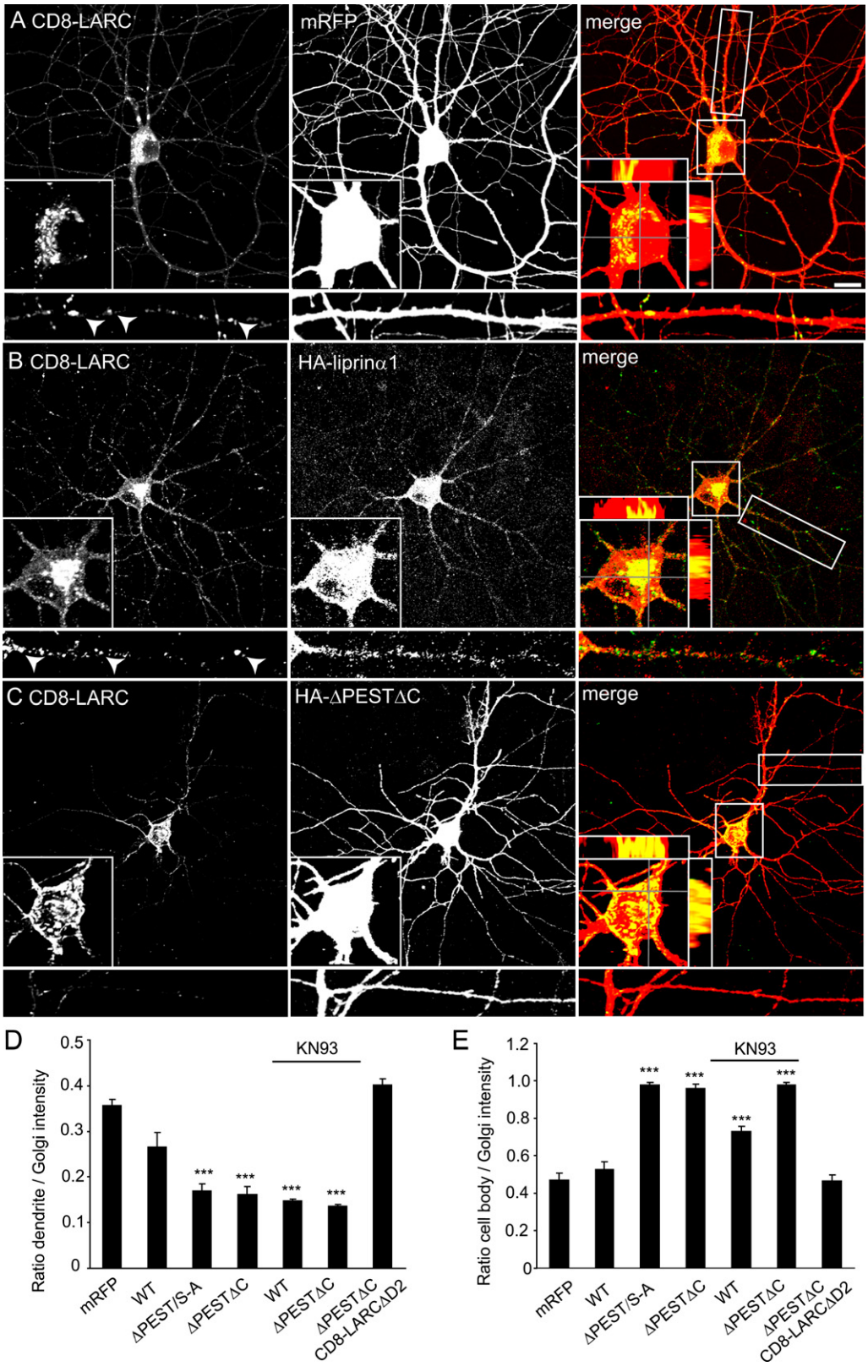
Antibodies and Reagents

Rabbit liprin (1069) antibody was previously described (Dunah et al., 2005; Wyszynski et al., 2002). Details of other antibodies and reagents are available in the Supplemental Data.

DNA Constructs

The following mammalian expression plasmids have been described: myc-liprinα1, myc-liprinα2, HA-liprinβ1 (Wyszynski et al., 2002), pSuper-Cdh1-shRNA (Konishi et al., 2004), pSuper-LAR-shRNA, and myr-HA-LAR-D2 (Dunah et al., 2005). CD8-LARC and CD8-LARCΔD2

Arrowheads indicate CD8-LARC localization in the perinuclear Golgi region (A, C, and D). Coexpression of liprinα1 (A) or liprinα1 mutants insensitive to CaMKII degradation (B) enhance surface expression of CD8-LARC. The scale bars represent 10 μm.
(E and F) HeLa cells transfected with CD8-LARC and control GFP (E) or GFP-liprinα1ΔPESTΔC (F) and immunostained for surface CD8 expression under nonpermeabilizing conditions. Liprinα1 mutants insensitive to CaMKII degradation enhance surface expression of CD8-LARC.


A CD8-LARC
mRFP
merge
B CD8-LARC
HA-liprinα1
merge
C CD8-LARC
HA-ΔPESTΔC
merge
D
Ratio dendrite / Golgi intensity
0.5
0.4
0.3
0.2
0.1
0
KN93
***
mRFP
WT
ΔPEST/S-A
ΔPESTΔC
WT
ΔPESTΔC
ΔPESTΔC
CD8-LARCΔD2
E
Ratio cell body / Golgi intensity
1.2
1.0
0.8
0.6
0.4
0.2
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KN93
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mRFP
WT
ΔPEST/S-A
ΔPESTΔC
WT
ΔPESTΔC
ΔPESTΔC
CD8-LARCΔD2

were generated by PCR cloning the intracellular C-terminal tail with and without the D2 domain of human LAR in frame with the extracellular and transmembrane domain of CD8 (Hoogenraad et al., 2005). CaMKII-shRNA sequences were targeted against rat CaMKIIα mRNA (GenBank accession number NM_012920) and rat CaMKIIβ mRNA (GenBank accession number NM_021739) corresponding to nucleotides 77–95 (CaMKIIα-shRNA) and 173–191 (CaMKIIβ-shRNA), respectively. APC2-shRNA sequences were targeted against rat APC2 (GenBank accession number BC107471; APC2-shRNA1: nucleotides 1554–1572 and APC2-shRNA2: nucleotides 1482–1500). For details of other constructs, see the Supplemental Data.

Primary Hippocampal Neuron Cultures, Transfection, and Immunocytochemistry

Primary hippocampal cultures were prepared from embryonic day 18 (E18) rat brains and transfected with various DNA constructs using Lipofectamine 2000 (Invitrogen) as described in the Supplemental Data. For immunocytochemistry, neurons were fixed for 10 min with 4% formaldehyde/4% sucrose in phosphate-buffered saline (PBS) at room temperature or ice-cold 100% methanol at −20°C, washed two times for 30 min with PBS at room temperature, and incubated with primary antibodies in GDB buffer (0.2% BSA, 0.8 M NaCl, 0.5% Triton X-100, 30 mM phosphate buffer [pH 7.4]) overnight at 4°C. Details of immunocytochemistry, image analysis, and quantification as well as immunoprecipitation and immunoblotting are available in the Supplemental Data.

Supplemental Data

Supplemental Data include seven figures and Supplemental Experimental Procedures and are available at http://www.developmentalcell.com/cgi/content/full/12/4/587/DC1/.

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Figure 7. Liprinα1 Mutants Insensitive to CaMKII Degradation Impair Dendritic Targeting of CD8-LARC in Hippocampal Neurons

(A–C) Representative images of hippocampal neurons cotransfected at DIV13 with CD8-LARC and mRFP (A), HA-liprinα1 (B), or HA-liprinα1ΔPESTΔC (C), and double-labeled with anti-CD8 (green) and anti-HA (red) antibodies. Dendritic segments (bottom) and cell body (insets; including confocal Z scans) are enlarged to show the distribution of CD8-LARC.

(D and E) Quantification of the ratio of immunostaining intensity in the dendrites (D) or cell body (E) versus the immunostaining intensity in the Golgi for CD8-LARC in neurons transfected with indicated constructs. "KN93" indicates overnight treatment with KN93. Histograms show mean ± SEM. *$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$. The scale bar represents 10 μm.

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