Regulation of the interaction between PIPKIγ and talin by proline-directed protein kinases

Sang Yoon Lee,[1,2] Sergey Voronov,[1,2] Kresimir Letinic,[1,3] Angus C. Nairn,[4] Gilbert Di Paolo,[1,2] and Pietro De Camilli[1,2]

[1]Howard Hughes Medical Institute and [2]Department of Cell Biology, [3]Department of Neurobiology, and [4]Department of Psychiatry, Yale University School of Medicine, New Haven, CT 06510

The interaction of talin with phosphatidylinositol(4) phosphate 5 kinase type Iγ (PIPKIγ) regulates PI(4,5)P_2 synthesis at synapses and at focal adhesions. Here, we show that phosphorylation of serine 650 (S650) within the talin-binding sequence of human PIPKIγ blocks this interaction. At synapses, S650 is phosphorylated by p35/Cdk5 and mitogen-activated protein kinase at rest, and dephosphorylated by calcineurin upon stimulation. S650 is also a substrate for cyclin B1/Cdk1 and its phosphorylation in mitosis correlates with focal adhesion disassembly. Phosphorylation by Src of the tyrosine adjacent to S650 (Y649 in human PIPKIγ) was shown to enhance PIPKIγ targeting to focal adhesions (Ling, K., R.L. Doughman, V.V. Iyer, A.J. Firestone, S.F. Bairstow, D.F. Mosher, M.D. Schaller, and R.A. Anderson. 2003. *J. Cell Biol.* 163:1339–1349). We find that Y649 phosphorylation does not stimulate directly PIPKIγ binding to talin, but may do so indirectly by inhibiting S650 phosphorylation. Conversely, S650 phosphorylation inhibits Y649 phosphorylation by Src. The opposite effects of the phosphorylation of Y649 and S650 likely play a critical role in regulating synaptic function as well as the balance between cell adhesion and cell motility.

Introduction

Phosphatidylinositol 4,5-bisphosphate (PI(4,5)P_2), a phosphoinositide highly enriched at the plasma membrane, plays a key role in intracellular signaling (De Camilli et al., 1996; Martin, 1998; Takenawa and Itoh, 2001; Yin and Janmey, 2003). The main pathway for the generation of PI(4,5)P_2 is phosphorylation of phosphatidylinositol(4)phosphate (PI(4)P) by the so-called type I PIP kinases, which function primarily as PI(4)P 5-kinases (Ishihara et al., 1998). The three catalytically active members of this family differ in cellular and subcellular distribution and, thus, control specific PI(4,5)P_2 pools (Doughman et al., 2003). PIP kinase type Iγ (PIPKIγ), which is expressed at high levels in the brain, comprises splice variants (PIPKIγ87 and PIPKIγ90) that differ because of the presence of a 28-aa tail (Ishihara et al., 1998). An additional splice variant that contains a 26-aa insert before the 28-aa tail was described recently (Giudici et al., 2004). Via the sequence WVYSPL contained within the 28-aa tail, PIPKIγ90 interacts with, and is activated by, talin (Di Paolo et al., 2002; Ling et al., 2002), which is an adaptor between the cytoplasmic domain of most β integrins (cell-surface receptors involved in cell adhesion) and the actin cytoskeleton (Calderwood and Ginsberg, 2003). Isoforms including the 28-aa tail predominate in brain, where they are concentrated at synapses (Di Paolo et al., 2002, 2004; Wenk et al., 2001). Accordingly, talin is present at synapses (Di Paolo et al., 2002; Morgan et al., 2004), and the relatively smaller pools of PIPKIγ90 present in nonneuronal cells are concentrated at focal adhesion sites (Di Paolo et al., 2002; Ling et al., 2002). Disruption of the talin–PIPKIγ90 interaction at synapses disrupts synaptic vesicle recycling and actin dynamics (Morgan et al., 2004), while its perturbation in nonneuronal cells affects cell adhesion (Di Paolo et al., 2002; Ling et al., 2002).

Binding of talin to the cytoplasmic domain of integrin β subunits induces conformational changes in the extracellular portion of the α/β integrin dimer leading to increased affinity for extracellular ligands (the so-called inside-out signaling) (Hynes, 2002; Calderwood and Ginsberg, 2003). Conversely, cell adhesion to substrates induces the nucleation of focal adhesions and the nucleation of the associated actin cytoskeleton. PI(4,5)P_2 is known to play an important regulatory role in this bidirectional signaling by its interactions with many proteins of focal adhesions, including talin (Martel et al., 2001; Takenawa and Itoh, 2001; Yin and Janmey, 2003). Talin binds to PIPKIγ90

Correspondence to Pietro De Camilli: pietro.decamilli@yale.edu

Abbreviations used in this paper: 2-D, two-dimensional; FERM, band 4.1/ezrin/radixin/moesin; ITC, isothermal titration calorimetry; PI(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; PIPKIγ, phosphatidylinositol(4)phosphate 5-kinase type Iγ.

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via its band 4.1/ezrin/radixin/moesin (FERM) domain, primarily by its F3 subdomain (Di Paolo et al., 2002; Ling et al., 2002), which also comprises the binding site for β integrin (Calderwood et al., 1999). In fact, the interactions of PIPKIγ90 and β integrin with the F3 subdomain are mutually exclusive (Barsukov et al., 2003). Accordingly, overexpression of constructs composing the 28-aa tail of PIPKIγ90 produces a "trans-dominant inhibition" of integrin function, by competing for its binding to talin (Calderwood et al., 2004). The tyrosine of the sequence WVYSPL undergoes endogenous phosphorylation by Src (Ling et al., 2003). This phosphorylation reaction, which may contribute to the stimulatory effect of focal adhesion kinase on focal adhesions, was reported to enhance the interaction between PIPKIγ90 and talin's F3 subdomain, which has a phosphotyrosine binding domain-like fold (Garcia-Alvarez et al., 2003).

PIPKIγ, in concert with the polyphosphoinositide phosphatase synaptojanin (Cremona et al., 1999), plays an important regulatory function in synaptic physiology. Based on genetic studies and other experimental approaches, it was proposed that PIPKIγ controls a plasma membrane pool of $PI(4,5)P_2$ implicated in synaptic vesicle exo-endocytosis (Wenk et al., 2001; Di Paolo et al., 2004) and that synaptojanin dephosphorylates $PI(4,5)P_2$ during the endocytic reaction (Cremona et al., 1999; Wenk and De Camilli, 2004). At synapses, both PIPKIγ (Wenk et al., 2001) and synaptojanin (Lee et al., 2004), as well as several other proteins implicated in vesicle recycling and actin function (Bauerfeind et al., 1997; Cousin and Robinson, 2001; Tan et al., 2003; Tomizawa et al., 2003), undergo constitutive phosphorylation and stimulation-dependent, Ca^{2+}-dependent dephosphorylation. At least for some of these proteins, phosphorylation at rest as well as rapid rephosphorylation after a depolarization stimulus is mediated by Cdk5 (Tan et al., 2003; Tomizawa et al., 2003; Lee et al., 2004). Conversely, dephosphorylation is mediated by the Ca^{2+}-dependent phosphatase calcineurin (Bauerfeind et al., 1997; Cousin and Robinson, 2001). In the case of synaptojanin 1, its dephosphorylation by calcineurin triggers its recruitment and activation at endocytic sites from a cytosolic pool (Lee et al., 2004).

The goal of the present study was to examine the regulation by phosphorylation of PIPKIγ90. Our results demonstrate that S650 within the talin-binding sequence WVYSPL is a main target of regulation by phosphorylation and that S650 phosphorylation blocks talin binding. Furthermore, a mutually antagonistic relationship exists between Src and proline-directed kinases with regard to the phosphorylation of adjacent sites on PIPKIγ90, thereby providing a mechanism for the bidirectional modulation of interactions between PIPKIγ90 and talin. This regulation is not restricted to synapses and may play an important and general function at focal adhesions.

Results

p35/Cdk5 phosphorylates PIPKIγ90 at serine 650

As a first step to determine whether Cdk5 may be responsible, at least in part, for the constitutive phosphorylation of PIPKIγ90 in resting synapses, we tested the ability of PIPKIγ90 to serve as a substrate for Cdk5 in vitro. Incubation of GST fusion protein of human PIPKIγ90 with purified p35/Cdk5 at an ~1:1 stoichiometric ratio resulted in its efficient phosphorylation (Fig. 1 A). Similar results were obtained with His_6-tagged PIPKIγ90 (Fig. 1 B), which was used for all subsequent phosphorylation experiments. p35, a prominent physiological substrate for the kinase activity of Cdk5 (Dhavan and Tsai, 2001), was also efficiently phosphorylated, as expected, and with similar kinetics during a 30-min incubation (Fig. 1 C). Two Cdk5 inhibitors, roscovitine (Tan et al., 2003) and butyrolactone I (Lee et al., 2004), inhibited the phosphorylation of both proteins (Fig. 1 A). A stoichiometric analysis revealed the incorporation of 2 mol phosphate/mol PIPKIγ90, which suggests at least two phosphorylation sites (Fig. 1 D).

To identify the residues phosphorylated by Cdk5, His_6-PIPKIγ90 was phosphorylated by p35/Cdk5 in the presence of

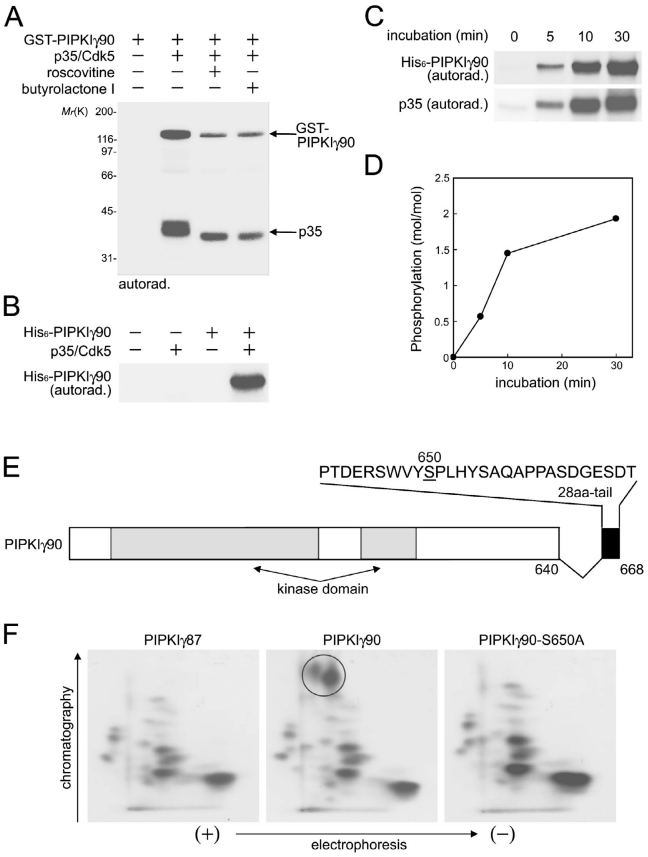

Figure 1. **PIPKIγ90 is phosphorylated by p35/Cdk5 at S650 in vitro.** Recombinant PIPKIγ90 fusion proteins were incubated with p35–Cdk5 complex in the presence of [^{32}P]ATP for 30 min. Protein phosphorylation was analyzed by autoradiography after SDS-PAGE. (A) GST-PIPKIγ90 was phosphorylated by p35/Cdk5 in the absence and presence of the Cdk5 inhibitors, roscovitine or butyrolactone I (20 μM each). (B and C) Phosphorylation of His_6-PIPKIγ90 by p35/Cdk5 and time course of the phosphorylation. (D) Stoichiometry of the phosphorylation of His_6-PIPKIγ90 by p35/Cdk5. Purified proteins phosphorylated in vitro in the presence of [^{32}P]ATP were separated by SDS-PAGE and stained with Coomassie brilliant blue. PIPKIγ90 bands were excised and incorporation of ^{32}P was measured. (E) Diagram indicating the position of the phosphorylation site S650 in the COOH-terminal 28-aa tail of PIPKIγ90. (F) 2-D phosphopeptide mapping of His_6-PIPKIγ87, His_6-PIPKIγ90, and S650A mutant His_6-PIPKIγ90 phosphorylated by p35/Cdk5 for 30 min in vitro. A circle outlines the spots present only in the protein that contains S650.

^{32}P[ATP] and subjected to trypsin fragmentation followed by HPLC separation. Matrix-assisted laser desorption ionization/mass spectrometry (MALDI/MS) analysis and Edman radiosequencing of the major radioactive peptide identified serine 650 (S650) (human sequence) as likely being a major site of phosphorylation. S650 is found within the sequence (S/T)PX(H/K/R) that fits the known Cdk5 consensus motif (Dhavan and Tsai, 2001). Notably, this sequence is part of the talin-binding site (Fig. 1 E; Di Paolo et al., 2002). The identification of S650 was confirmed by two-dimensional (2-D) phosphopeptide mapping of in vitro–phosphorylated His_6-PIPKIγ90 and His_6-PIPKIγ87 (Fig. 1 F), which differ only by the presence or absence of the 28-aa tail. Although several identical radioactive peptides were observed for both isoforms, one major peptide and an adjacent accessory peptide (Fig. 1 F, circled region) were observed only for PIPKIγ90 (Fig. 1 F). Phosphorylation of these two peptides saturated more rapidly than the other peptides based on comparison of autoradiograms obtained from 5-, 30-, and 60-min phosphorylation reactions (unpublished data). When S650 was mutated to alanine (S650A), these peptides were no longer observed. These data indicate that, at least in vitro, S650 is the most rapidly phosphorylated, although not the only, site for Cdk5 in PIPKIγ90. Two other sites, serine 453 and threonine 123, that are common to PIPKIγ90 and PIPKIγ87 fit the Cdk5 consensus motif. Preliminary experiments indicate that Cdk5 may phosphorylate S453; in contrast, mutation of T123 to alanine did not alter the 2-D phosphopeptide mapping pattern (unpublished data). In this study, we have focused on S650 because of its location within the minimal talin-binding motif (WVYSPL).

To further confirm the site of phosphorylation and for use in the studies described later, an antibody that specifically recognizes PIPKIγ90 phosphorylated at S650 (anti–phospho-S650 [pS650] antibody) was raised. The pS650 antibody selectively recognized WT PIPKIγ90 phosphorylated in vitro by p35/Cdk5 (Fig. 2 A). Under the same conditions (after 30-min incubation), the S650A mutant was phosphorylated to a much lower extent as revealed by the incorporation of ^{32}P (reflecting one or other phosphorylation sites besides S650), and no signal was detected with the pS650 antibody (Fig. 2 A). WT or S650A HA-PIPKIγ90 was transfected into CHO cells with p35 plus Cdk5. Using the pS650 antibody to analyze immunoprecipitated PIPKIγ90, the WT, but not the S650 mutant, protein was found to be phosphorylated (Fig. 2 B). Omission of p35 and Cdk5, or transfection with catalytically inactive mutant of Cdk5 (mut-Cdk5; Patrick et al., 1999) resulted in a significantly lower phosphorylation of S650 compared with that observed after transfection with p35/Cdk5 (Fig. 2 C). Under these conditions, S650 may be phosphorylated by low levels of endogenous Cdk5 (Dhavan and Tsai, 2001) and/or by other proline-directed protein kinases (see Figs. 7 and 8).

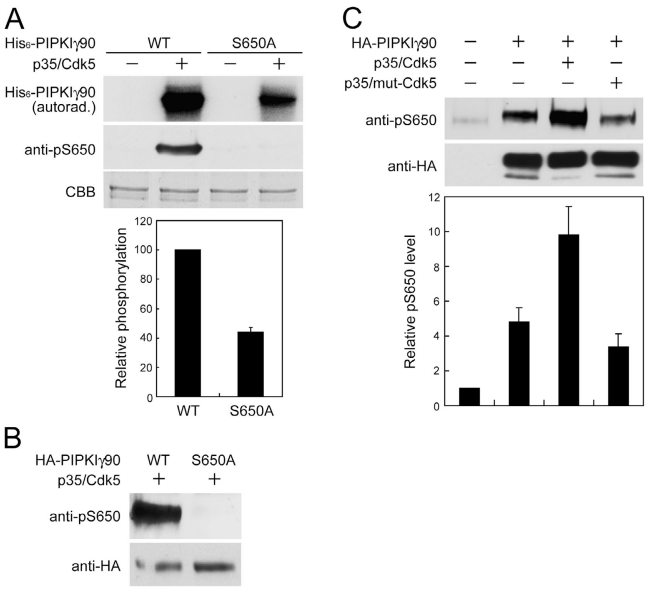

Figure 2. **PIPKIγ90 is phosphorylated by p35/Cdk5 at S650 in vivo.** (A) Generation and characterization of a polyclonal antibody specific for phospho-S650 (pS650). Purified WT and S650A mutant His_6-PIPKIγ90 were incubated with or without p35/Cdk5 and with either nonradioactive ATP (for Western blotting) or [^{32}P]ATP (for autoradiography) for 30 min. Samples were analyzed by autoradiography, and Western blotting with the anti-pS650 antibody was performed. Coomassie brilliant blue (CBB) staining shows loading of equal amounts of substrate proteins. The autoradiographic signal was quantified by a phosphorimager analysis and values were expressed as a percentage of the radioactivity incorporated into the WT sample. Bar graphs represent mean ± SD (n = 3). (B) CHO cells were transfected with WT or S650A mutant HA-PIPKIγ90 together with p35 and Cdk5. PIPKIγ90 immunoprecipitates were obtained from cell lysates 24 h after transfection. S650 phosphorylation and PIPKIγ90 expression were analyzed by Western blotting with anti-pS650 and anti-HA antibodies, respectively. (C) CHO cells were cotransfected with HA-PIPKIγ90, p35, and Cdk5 or mut-Cdk5. PIPKIγ90 immunoprecipitates from transfected and control cells were analyzed by Western blotting as described above. The faint band visible in nontransfected CHO cells may represent phosphorylated hamster PIPKIγ90. The pS650 immunoreactivity was quantified using an NIH image analysis software and shown as mean ± SD (n = 3).

Phosphorylation of S650 inhibits the interaction of PIPKIγ90 with talin in vitro and in vivo

Given the location of S650 in the middle of the talin-binding sequence (WVYSPL; Di Paolo et al., 2002), the possibility that phosphorylation of S650 of PIPKIγ90 inhibits its interaction with talin was next examined. Isothermal titration calorimetry (ITC) was used to analyze the binding of the GST-talin head (Di Paolo et al., 2002), which comprises the FERM domain, to synthetic 12-mer dephospho- and phosphopeptides encompassing the talin-binding sequence of PIPKIγ90. K_d values of 2 μM and >1 mM were observed for the dephosphopeptide and the pS650 peptide, respectively (Fig. 3 A), indicating a strongly negative role of S650 phosphorylation in talin binding. A K_d of 2 μM (see Fig. 6 A) value for the WT peptide is in agreement with the dissociation constant of the same interaction measured with tryptophan fluorescence by Barsukov et al. (2003), but is higher than the dissociation constant observed by Ling et al. (2003) using fluorescence anisotropy measurement. Differences in the assays and the experimental conditions may explain the discrepancy. For example, our values were obtained at 37°C, whereas the value of Ling et al. (2003) was recorded at room temperature.

We further examined whether the in vivo interaction of PIPKIγ90 with talin is inhibited by Cdk5-dependent phosphorylation. CHO cells were transfected with HA-PIPKIγ90, p35,

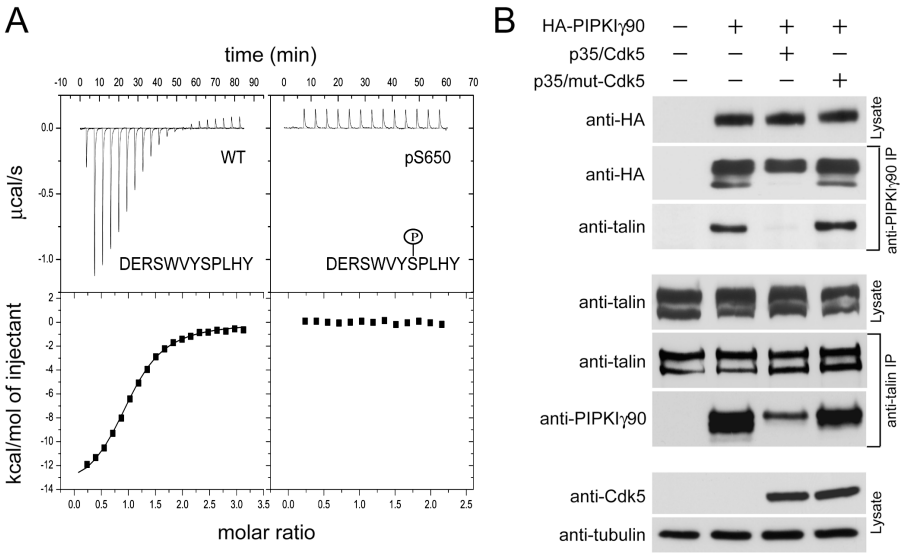

Figure 3 **Cdk5 phosphorylation of PIPKIγ90 inhibits its interaction with talin in vivo.** (A) ITC analysis of the binding of 12-mer WT and pS650 peptides from the 28-aa tail of PIPKIγ90 to GST-talin head. Raw data as a function of time are shown in the top panels, and plots of the total heat released as a function of the molar ratio of each ligand are shown in the bottom panels. The continuous line in the bottom panels represents the nonlinear, least-squares best fits to the experimental data using a one-site model of binding. Note the roughly 1:1 stoichiometry indicated by the half-height point of the sigmoidal curve (bottom left; Turnbull and Daranas, 2003), and the complete absence of heat release in the case of the pS650 peptide. (B) CHO cells were cotransfected with HA-PIPKIγ90, p35, and Cdk5 or mut-Cdk5. Protein contents of the starting lysates were revealed by Western blotting. PIPKIγ90 and talin were immunoprecipitated from the cell lysates and presence of PIPKIγ90 and talin in each immunoprecipitate was detected by Western blotting.

and either Cdk5 or mut-Cdk5 (as in Fig. 2 C). Cell lysates were then processed for anti-PIPKIγ90 or antitalin immunoprecipitation, and the starting lysates and the resulting pellets were analyzed by Western blotting for transfected and control proteins (Fig. 3 B). Talin coprecipitated with HA-PIPKIγ90, and HA-PIPKIγ90 coprecipitated with talin, as expected. However, expression of Cdk5, but not mut-Cdk5, strongly inhibited the coprecipitation of the two proteins.

We next determined whether a phosphomimetic mutation of S650 to aspartic acid (S650D) affected the interaction with talin. WT and S650D His_6-PIPKIγ90 were overlaid in a "far-Western" assay with a GST fusion protein of the F3 subdomain of the FERM domain of talin (Fig. 4, A and C). The F3 domain clearly bound to the WT protein, as expected (Di Paolo et al., 2002), but no binding was observed for the S650D mutant (Fig. 4 A). Binding of the F3 domain to the S650A mutant was also reduced, although not abolished, and this is in agreement with a potential role of the side chain of serine in the binding (Di Paolo et al., 2002; Liddington et al., 2003). These results were qualitatively confirmed by His_6-PIPKIγ90 pull-down assays

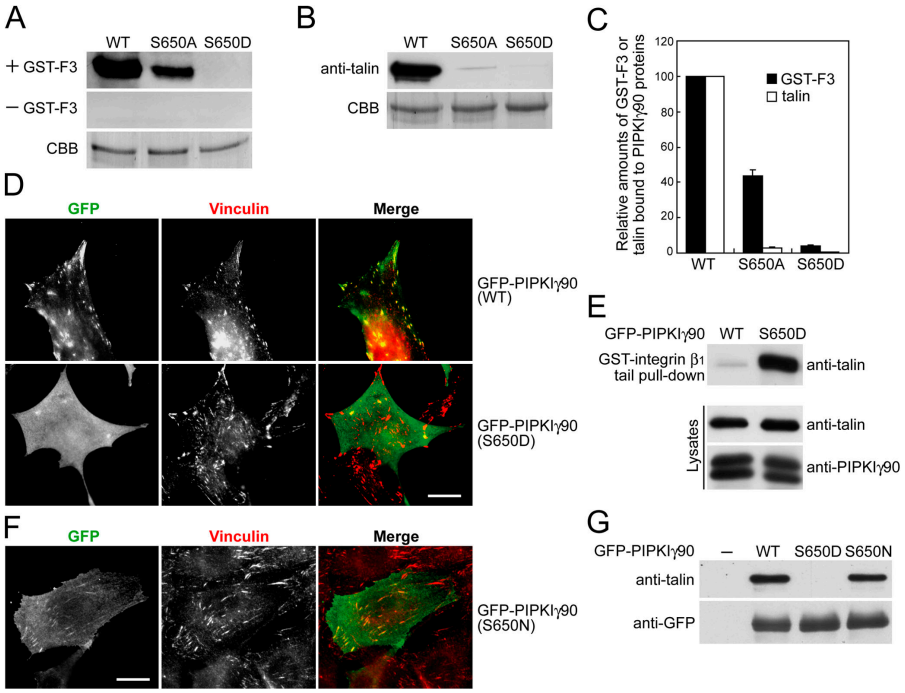

Figure 4. **Phosphomimetic mutation at S650 (S650D) disrupts the interaction of PIPKIγ90 with talin in vitro.** (A) GST-F3 overlay assay. Nitrocellulose blots of WT and mutant His_6-PIPKIγ90 were overlaid with or without GST-F3 fusion protein, and then overlaid with anti-GST antibody. CBB staining reveals equal load of the lanes. (B) Pull-down assay from rat brain extracts on bead-immobilized WT and mutant His_6-PIPKIγ90. Bound talin was revealed by Western blotting, and equal amount of bait proteins was revealed by CBB staining. (C) The intensities of the GST-F3 and talin bands shown in A and B were quantified by an NIH image analysis software. Values from mutant proteins were normalized to that from WT and are represented as mean ± SD (n = 4). (D) Localization of transfected GFP-PIPKIγ90. NIH3T3 cells were transfected with GFP-PIPKIγ90 or its S650D mutant, and then were processed by immunofluorescence for vinculin immunoreactivity. Bar, 10 µm. (E) Pull-down assay from lysates of CHO cells transfected with WT and S650D mutant GFP-PIPKIγ90. Bead-immobilized GST-integrin β_1 tail was used as a bait. Talin and PIPKIγ90 in the lysates or bead fractions were detected by Western blotting. (F) NIH3T3 cells transfected with mutant (S650N) GFP-PIPKIγ90 were immunostained with antivinculin antibody. Bar, 10 µm. (G) CHO cells were transfected with WT, S650D, or S650N GFP-PIPKIγ90. After 24 h of transfection, cell lysates were immunoprecipitated with anti-PIPKIγ90 antibody, and the presence of talin and PIPKIγ90 in the immunoprecipitates was analyzed by Western blotting.

from rat brain extracts (Fig. 4, B and C). In this case, the loss of binding produced by the S650A mutation was nearly as large as that produced by the S650D mutation.

The effect of the phosphomimetic S650D mutation on the properties of PIPKIγ90 was further investigated in living cells. WT, but not mutant (S650D) GFP-PIPKIγ90, accumulated at focal adhesions when expressed in NIH3T3 cells (mouse fibroblast), as shown by colocalization with the focal adhesion marker vinculin (Fig. 4 D). This result demonstrates the inhibitory effect of the presence of an acidic charge at position 650 in the recruitment of PIPKIγ90 at focal adhesions, which is consistent with the blocking effect on the interaction with talin. As reported previously, the interaction of talin with PIPKIγ90 competes with the interaction of talin with β integrin, because both proteins bind to the same surface of talin's F3 domain (Barsukov et al., 2003; Liddington et al., 2003; Calderwood et al., 2004). Accordingly, pull-downs with a GST fusion protein of integrin β_1 tail from lysates of CHO cells transfected with mutant (S650D) or wild-type PIPKIγ90 yielded a higher level of talin in the case of cells transfected with the mutant protein, which is not expected to compete (Fig. 4 E). Collectively, these results indicate that talin binding is incompatible with the presence of a negative charge at S650 in PIPKIγ90 and that S650 phosphorylation has an important role in the regulation of PIPKIγ90–talin–integrin interactions.

PIPKIγ90 and β integrin share amino acid similarity in the talin-binding region. Within these similar sequences, S650 of PIPKIγ90 was proposed to functionally replace the asparagine of the sequence NPXY in β integrin. When S650 of PIPKIγ90 was replaced by an asparagine (S650N), the resulting mutant protein was still able to bind to talin, confirming this prediction. Thus, transfected GFP-PIPKIγ90 harboring the S650N mutation colocalized with vinculin at focal adhesions (Fig. 4 F) and coprecipitated with talin from lysates of transfected cells (Fig. 4 G). The presence of a serine at this position in PIPKIγ90, but not in β integrin, allows for a differential regulation of the two interactions by phosphorylation.

If the binding of PIPKIγ90 to talin is important for focal adhesion dynamics, overexpression of p35/Cdk5 would be expected to affect focal adhesion dynamics by enhancing the phosphorylation state of S650. Consistent with this prediction, overexpression of p35/Cdk5 in NIH3T3 cells disrupted focal adhesions and stress fibers, as detected by antivinculin immunostaining and phalloidin staining, respectively (Fig. 5).

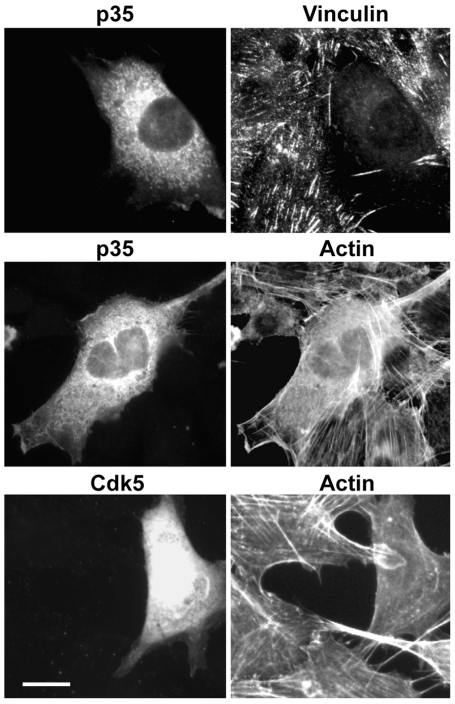

Figure 5. **Inhibitory effects of p35/Cdk5 overexpression on focal adhesion.** NIH3T3 cells were cotransfected with both p35 and Cdk5 for 24 h, and the overexpressed proteins were detected by immunofluorescence microscopy with the antibodies indicated. Focal adhesions were visualized by antivinculin immunostaining, and actin was visualized by fluorescent phalloidin. Bar, 10 μm.

PIPKIγ90 phosphorylation by Cdk5 and Src is mutually exclusive

Ling et al. (2003) reported that the COOH-terminal region of mouse PIPKIγ90 undergoes phosphorylation by Src at tyrosine 644 (Y649 of human PIPKIγ90) and that this phosphorylation enhances its interaction with talin. Y649 is adjacent to S650 in the talin-binding sequence (WVYSPL), which is identical in mice and humans. The juxtaposition of the two sites raised the possibility that phosphorylation of one might regulate phosphorylation of the other. This possibility was tested using synthetic peptides centered around the talin-binding consensus. Both 12-mer peptides, as well as 17-mer peptides identical to those used by Ling et al. (2003), were tested. First, we revisited the effect of Y649 phosphorylation on talin binding. Surprisingly, an ITC assay did not demonstrate a difference between the affinities of the WT peptides and of the pY649 peptides for GST-talin head (Fig. 6 A). Phosphorylation of tyrosine 654, which is a weak Src target site (Ling et al., 2003), also had no positive effect on talin binding, and in fact, it slightly decreased the binding (Fig. 6 A). The pS650 12-mer peptide exhibited negligible binding to talin, as expected (Figs. 6 A and 3 A). In agreement with the results of the ITC assay, the WT peptide (12-mer) and the pY649 peptide (12-mer) equally competed with the tail for talin binding in pull-down assays from rat brain extracts using GST–28-aa tail (unpublished data).

Next, we tested whether the presence of a phosphate at position 649 or 650 affected the phosphorylation of the adjacent site. The 12-mer WT, pY649, and pS650 peptides were incubated under linear rate conditions with purified c-Src or p35/Cdk5 and ^{32}P[ATP]. The pY649 peptide was a poor substrate for Cdk5 (Fig. 6 B). Likewise, the pS650 peptide was a poor substrate for c-Src. As expected, the pY649 and pS650 peptides were hardly phosphorylated by c-Src and Cdk5, respectively. These data suggest that phosphorylation of either site has a strong inhibitory effect on the phosphorylation of the other site.

S650 phosphorylation of PIPKIγ90 is regulated by Cdk5 and calcineurin at synapses

In the next series of experiments, the in vivo phosphorylation of S650 in endogenous PIPKIγ90 was investigated. Rat brain syn-

A

peptides	Kd (μM)	ΔH (kcal/mole)
WT (17-mer)	1.7 ± 0.1	-11.0 ± 0.1
pY649 (17-mer)	1.7 ± 0.2	-10.7 ± 0.2
pY654 (17-mer)	3.9 ± 0.3	-10.9 ± 0.1
WT (12-mer)	2.0 ± 0.2	-14.6 ± 0.3
pY649 (12-mer)	3.0 ± 0.2	-11.8 ± 0.1
pS650 (12-mer)	N/A, >1000	N/A

WT (17-mer) PTDERSWVYSPLHYSAR
pY649 PTDERSWVpYSPLHYSAR
pY654 PTDERSWVYSPLHpYSAR

WT (12-mer) DERSWVYSPLHY
pY649 DERSWVpYSPLHY
pS650 DERSWVYpSPLHY

B

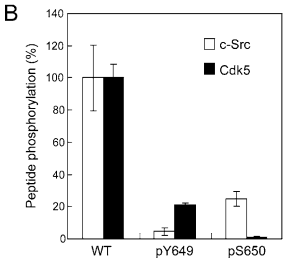

Figure 6. **Phosphorylation of either Y649 or S650 inhibits the phosphorylation of the adjacent site.** (A) Binding of PIPKIγ90 peptides to GST-talin head as determined by ITC. Note that both the dissociation constant (K_d) and the enthalpy (ΔH) of the binding are similar for WT and pY649 peptides. The pY654 peptide has lower affinity, but the pS650 peptide did not bind. N/A, not available. (B) In vitro phosphorylation by Cdk5 and c-Src of WT, pY649 and pS650 12-mer PIPKIγ90 peptides. Each peptide was incubated in the presence of [^{32}P]ATP and of either p35/Cdk5 or c-Src for 20 min at 30°C. Peptides were then recovered on phosphocellulose paper, and the associated radioactivity was measured by Cerenkov counting. Incorporation of radioactivity into pY649 and pS650 peptides was normalized to that of the WT peptide and presented as mean ± SEM (n = 4)

aptosomes, including samples exposed to a 1-h metabolic labeling step with ^{32}Pi, were incubated for 20 min in control buffer, and then were depolarized for 1 min with high K^+ (55 mM) in the absence or presence of extracellular Ca^{2+}. As detected by using ^{32}P incorporation or by using the pS650 antibody, we found that PIPKIγ90 was constitutively phosphorylated at rest, and dephosphorylated upon high K^+ stimulation, but only in the presence of extracellular Ca^{2+} (Fig. 7 A). The stimulation-dependent dephosphorylation of pS650 was blocked by cyclosporin A, a calcineurin inhibitor (Fig. 7 B). Furthermore, rephosphorylation of S650 upon reexposure to the control buffer was partially inhibited by incubation with butyrolactone I (Fig. 7 C) or roscovitine (unpublished data). The pattern of PIPKIγ90 dephosphorylation was qualitatively similar to that of amphiphysin 2, whose upper band (phospho-form) collapses into lower bands (dephospho form) upon calcineurin-dependent dephosphorylation (Fig. 7 A; Bauerfeind et al., 1997; Cousin and Robinson, 2001).

The only partial effect of Cdk5 inhibitors on the phosphorylation of S650 suggested that other kinases may phosphorylate this site. Interestingly, the phosphorylation/dephosphorylation of S650 in resting and stimulated synaptosomes was reminiscent of that of the phosphorylation sites 4 and 5 of synapsin I (corresponding to serine 62 and serine 67, respectively, of rat synapsin I) (Fig. 7 B; Jovanovic et al., 2001). Notably, these sites in synapsin I were found to be phosphorylated by MAPK (Jovanovic et al., 2001). MAPK is a proline-directed serine/threonine kinase whose substrate specificity [(S/T)P] (Songyang et al., 1996) overlaps with the substrate preference of Cdk5 and may therefore be involved in S650 phosphorylation.

The potential involvement of MAPK as well as of phosphatases other than calcineurin in the regulation of S650 phosphorylation was investigated. Synaptosomes were treated with either okadaic acid, an inhibitor of protein phosphatase 2A (PP2A), or PD98059, an inhibitor of MAPK1/2 (p42/p44). Okadaic acid alone strongly enhanced the phosphorylation state of S650 both at rest and after high K^+ depolarization (Fig. 7 D), although a stimulation-dependent dephosphorylation of S650 still occurred upon exposure to high K^+ (Fig. 7 D, compare the second and fourth lanes). Okadaic acid also increased the level of phospho-MAPK1/2 (Fig. 7 D). This was expected because PP2A is known to dephosphorylate MAPK, with a resulting inhibition of its activity (Alessi et al., 1995). PD98059 partially prevented the increase in S650 phosphorylation produced by okadaic acid (Fig. 7 E). The increase of pS650 produced by okadaic acid may result both from a direct inhibition of pS650 dephosphorylation by PP2A and/or indirectly from the enhanced MAPK activity caused by inhibition of PP2A. Note that, as previously reported, depolarization in the presence of extracellular Ca^{2+} increases the phosphorylation state of MAPK1/2 (Fig. 7 D, compare the first and third lanes; Yamagata et al., 2002). This is in contrast to the depolarization-dependent dephosphorylation of S650, thus indicating that calcineurin can override the action of MAPK on S650. Consistent with PIPKIγ90 being a substrate for MAPK, purified MAPK1 phosphorylated S650 in WT His_6-PIPKIγ90 in vitro, but did not phosphorylate S650A mutant His_6-PIPKIγ90 (Fig. 7 F), as determined by anti-p5650 Western blotting.

S650 of PIPKIγ90 undergoes mitotic phosphorylation

Entry of cells into mitosis is accompanied by massive structural changes. For example, cells grown in culture round up and partially detach from the substrates, a change which correlates with the disruption of focal adhesions (Maddox and Burridge, 2003). The property of S650 of PIPKIγ90 to function as a substrate for Cdk5, a member of the Cdk family, prompted us to examine its phosphorylation state during mitosis. Disruption of the interaction between talin and PIPKIγ90 could be one of the biochemical modifications that correlate with focal adhesion disassembly. CHO cells transfected with WT or mutant HA-PIPKIγ90 were either treated with nocodazole to arrest cells in pro-metaphase, or treated with nocodazole and then allowed to progress to G_1 interphase by re-addition of serum. pS650 immunoreactivity was detected only in cells expressing WT PIPKIγ90 and phosphorylation was dramatically increased in mitotic cells (Fig. 8 A). These results were corroborated by the analysis of endogenous PIPKIγ90 in cells (U87MG cells, derived from a human astrocytoma) that express high levels of this enzyme. In this case, cell lysates were subjected to immunoprecipitation with anti-PIPKIγ90 antibody prior to Western blotting (Fig. 8 B). Not only S650 phosphorylation was strongly stimulated in mitosis, but coprecipitation of talin with

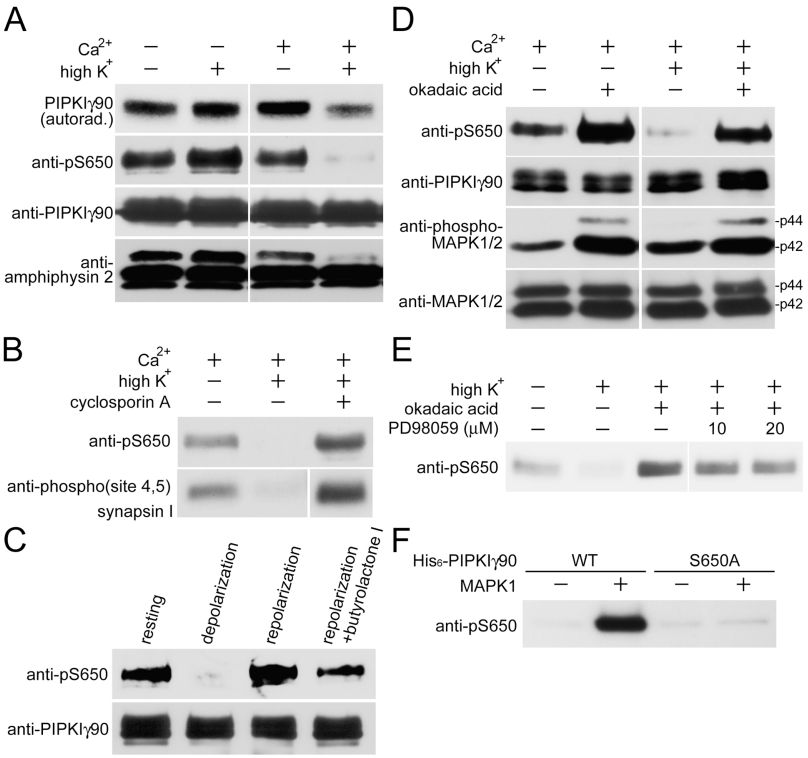

Figure 7. **Regulation of S650 phosphorylation in synaptosomes.** In all panels shown in the figure, with the exception of E and F, levels of total PIPKIγ90 and of its pS650 epitope were analyzed by Western blots of anti-PIPKIγ90 immunoprecipitates obtained from synaptosomal lysates. For other proteins, Western blots were performed directly on synaptosomal lysates. (A) Freshly prepared rat brain synaptosomes, or synaptosomes exposed to a 1-h labeling step with ^{32}Pi, were incubated for 1 min with either control buffer or stimulation buffer (high K^+) in the absence or presence of Ca^{2+} (Bauerfeind et al., 1997). Anti-PIPKIγ90 immunoprecipitates prepared from these samples were analyzed by autoradiography (^{32}P-labeled samples) or by Western blotting for pS650 and total PIPKIγ90. Western blotting for amphiphysin 2 revealed the previously described stimulation-dependent mobility shift of the upper band (because of its dephosphorylation), thus confirming the occurrence of Ca^{2+}-dependent stimulation. (B) Synaptosomes were stimulated with high K^+ for 1 min in the absence or presence of 2 μM cyclosporin A, and then were analyzed with antibodies directed against pS650 or phospho-sites 4 and 5 (MAPK sites) of synapsin I. (C) Synaptosomes were exposed for 1 min to either control buffer (resting) or high K^+ buffer (depolarization). Aliquots of stimulated synaptosomes were then returned for 15 min to control buffer (repolarization) with and without the Cdk5 inhibitor butyrolactone I (10 μM). (D) Synaptosomes were incubated for 1 min with control buffer or high K^+ buffer in the absence or presence of 1 μM okadaic acid, and then were analyzed for levels of pS650, PIPKIγ90, phospho-MAPK1/2, and total MAPK1/2. (E) Synaptosomes were stimulated for 1 min in the absence or presence of 1 μM okadaic acid and the MAPK inhibitor PD98059. (F) In vitro phosphorylation of WT and S650A mutant His_6-PIPKIγ90 with purified MAPK1. S650 phosphorylation by MAPK1 was detected by the anti-pS650 antibody.

the kinase was nearly abolished in the mitotic state. The mitotic synchronization of these cells was validated by Western blotting of total lysates with antibodies directed against phosphohistone H3, a marker of mitosis (Ajiro et al., 1996). In support of a role for cyclin-activated kinases in the phosphorylation of PIPKIγ90 during mitosis, purified cyclin B1–Cdk1 complex phosphorylated purified WT His_6-PIPKIγ90 in vitro but weakly phosphorylated its S650A mutant (Fig. 8 C). Cyclin B1, a physiological substrate of Cdk1 (Borgne et al., 1999), was phosphorylated at roughly similar levels in both samples (Fig. 8 C).

Discussion

We and others have recently identified an interaction between talin and PIPKIγ90 that plays an important role at synapses and focal adhesions (Di Paolo et al., 2002; Ling et al., 2002). This interaction is thought to control the synthesis of specific pools of $PI(4,5)P_2$ at these sites, but has an additional impact on adhesion. PIPKIγ90 competes with the binding of integrin to talin and can, therefore, transdominantly inhibit integrin activation (Barsukov et al., 2003; Calderwood et al., 2004). Thus, the binding of PIPKIγ90 to talin must be highly regulated. Ling et al. (2003) suggested one mechanism for positive regulation as being the Src-dependent phosphorylation of Y649 within the talin-binding sequence WVYSPL. We have now identified and characterized another mechanism, phosphorylation of S650, that negatively regulates the talin–PIPKIγ90 interaction and that is the point of convergence of multiple signaling pathways. In addition, our results suggest that Src-mediated phosphorylation of Y649 likely acts indirectly on talin binding, rather than directly, as previously proposed (Ling et al., 2003).

At synapses, the generation of $PI(4,5)P_2$ by PIPKIγ is thought to play an important regulatory role both in exocytosis and in endocytosis (Wenk et al., 2001; Di Paolo et al., 2004; Wenk and De Camilli, 2004). The initial goal of this study was to determine whether PIPKIγ90, like several other synaptic proteins that participate in synaptic vesicle traffic, primarily endocytic traffic (Bauerfeind et al., 1997; Cousin and Robinson, 2001; Tan et al., 2003; Tomizawa et al., 2003; Lee et al., 2004), is regulated by the serine/threonine phosphorylation. We have now demonstrated that PIPKIγ90 is efficiently phosphorylated at S650 by Cdk5 both in vitro and in vivo, and that this Cdk5 site is dephosphorylated by calcineurin. Importantly, we have shown that phosphorylation of this site negatively regulates the talin–PIPKIγ90 interaction. Such an interaction is vital for synaptic physiology, because its perturbation by peptides microinjected into giant axons perturbs synaptic vesicle recycling and the dynamics of presynaptic actin (Morgan et al., 2004). Restoration of the interaction by depolarization-triggered dephosphorylation may serve to recruit and activate PIPKIγ90 at sites of exo-endocytosis and may underlie, together with interaction of PIPKIγ90 with small GTPases (Aikawa and Martin, 2003; Krauss et al., 2003), the increase in $PI(4,5)P_2$ synthesis occurring in stimulated synaptosomes (Di Paolo et al., 2004). Interestingly, in preliminary experiments, we have found that a guanyl nucleotide–independent interaction of PIPKIγ90 with Rac1 is also inhibited by Cdk5-depen-

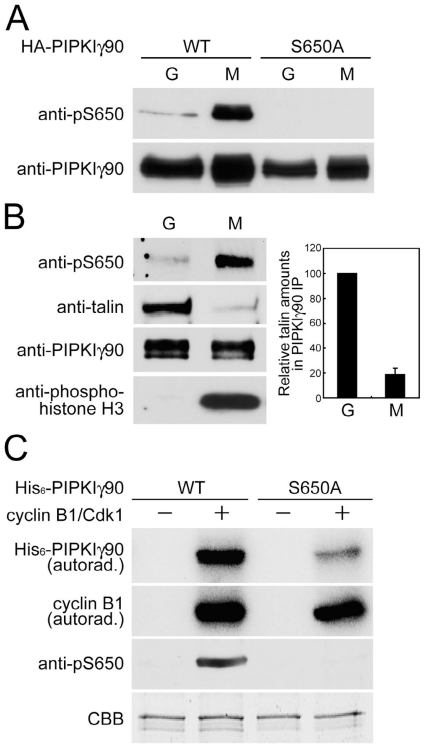

Figure 8. **S650 of PIPKIγ90 undergoes mitotic phosphorylation by cyclin B1/Cdk1.** (A) CHO cells transfected with WT or S650A mutant HA-PIPKIγ90 for 24 h were arrested in the mitotic state (M) by nocodazole treatment. G_1 interphase cells (G) were further prepared from mitotically synchronized cells after removal of nocodazole. Cell lysates of both M and G cells were analyzed by Western blotting with anti-pS650 and anti-PIPKIγ90 antibodies. (B) U87MG cells were processed as described in A to generate mitotic and interphase cells. PIPKIγ90 was immunoprecipitated from cell lysates for analysis of pS650, talin and total PIPKIγ90. Cell lysates were also analyzed by Western blotting for levels of histone H3 phosphorylation using a phosphospecific antibody. Bar graphs represent normalized talin immunoreactivity (mean ± SD; $n = 4$) after quantification as shown in Fig. 4 C. (C) WT and S650A mutant His_6-PIPKIγ90 were incubated in vitro with or without the cyclin B1–Cdk1 complex in the presence of [^{32}P]ATP. His_6-PIPKIγ90 phosphorylation and cyclin B1 autophosphorylation were examined by autoradiography after SDS-PAGE. In parallel, samples phosphorylated under the same conditions with nonradioactive ATP were processed for Western blotting with anti-pS650 antibody. CBB staining demonstrates equal amounts of proteins.

dent phosphorylation at a site distinct from S650 (unpublished data). These findings support a model according to which Cdk5 phosphorylation keeps the synapse in a "resting mode," whereas calcineurin triggers an "active mode" (Lee et al., 2004; Sahin and Bibb, 2004).

Our results suggest that phosphorylation of S650 may also be regulated by other proline-directed kinases and at least one other phosphatase. A potential role of MAPK can be inferred from the inhibitory action of the MAPK inhibitor PD98059 on the levels of pS650 in vivo and from the in vitro phosphorylation of PIPKIγ90 by purified MAPK1. The powerful stimulatory effect of okadaic acid, an inhibitor of the PP2A (Jovanovic et al., 2001), on the levels of pS650 is also consistent with an action of MAPK, because PP2A could act indirectly by dephosphorylating and, therefore, inhibiting MAPK (Alessi et al., 1995). In the presence of okadaic acid, such activity would be greatly stimulated. Additionally, PP2A may act directly on pS650. If this is the case, the large increase of pS650 observed in the presence of okadaic acid in unstimulated synaptosomes would indicate the occurrence of a very rapid turnover of phosphate on S650 even at rest. Among the questions that remain to be addressed is whether different kinases and phosphatases act on pools of PIPKIγ90 with distinct subcellular localization, because PIPKIγ90 is concentrated in, but not restricted to, nerve terminals. However, important conclusions from this study are that S650 represents the point of convergence of different regulatory pathways and that its state of phosphorylation controls an important targeting and activation mechanism of this enzyme.

Cdk5 phosphorylation was found to have only a negligible (positive) effect on the catalytic activity of PIPKIγ90 (unpublished data). Because Cdk5 also phosphorylated PIPKIγ87 and slightly activated its catalytic activity (unpublished data), S650 does not seem to participate in this regulation. However, it is possible, that sites phosphorylated by other kinases may directly control its catalytic activity. An inhibitory role on PIPKIγ activity produced by protein kinase A phosphorylation at serine 264 (human sequence) was previously suggested, based on studies of the homologous mouse PIPKIα (Park et al., 2001). The precise coordination of different phosphorylation reactions remains to be investigated.

Our study also shows that S650 of PIPKIγ90 can undergo phosphorylation in nonneuronal cells, where PIPKIγ90 is primarily localized at focal adhesions. However, although transfected Cdk5—a protein kinase expressed predominantly, but not exclusively, in neurons (Dhavan and Tsai, 2001)—can phosphorylate transfected PIPKIγ90 in CHO cells, the protein kinase that performs this phosphorylation reaction physiologically in interphase nonneuronal cells remains to be identified. As suggested by the phosphomimetic S650D mutant, phosphorylation of S650 prevents the localization of PIPKIγ90 at focal adhesions. In addition, the S650D mutant of PIPKIγ90 did not compete with the interaction of talin and β integrin. Therefore, one can expect that regulation of the turnover of phosphate on S650 may have a key role in the regulation of the balance between adhesion and motility (Calderwood and Ginsberg, 2003; Carragher and Frame, 2004). It is of interest, in this context, that Cdk5 plays a major role in the regulation of cell migration and neurite outgrowth in the nervous system (Dhavan and Tsai, 2001; Xie et al., 2003) and of cell adhesion and migration in nonneuronal cells (Gao et al., 2002). As we have shown here, its overexpression strongly affects focal adhesion.

As we also show here, S650 of PIPKIγ90 undergoes phosphorylation in mitosis and is a very good in vitro, and a likely in vivo, substrate for the cyclin B1–Cdk1 complex. It will be of interest to determine whether phosphorylation of PIPKIγ90 at S650 helps drive focal adhesion disassembly during mitosis, or simply correlates with this process. We note that other proteins implicated in membrane traffic at synapses are also physiological substrates for both cyclin B1/Cdk1 and p35/Cdk5. These include epsin, Eps15, and amphiphysin (Chen et al., 1999; Floyd et al., 2001). Mechanisms that regulate entry into the mitotic state, where much of the exo-endocytic traffic is blocked (Py-

paert et al., 1991), may be closely related to mechanisms that limit exo-endocytic traffic at neuronal synapses at rest.

At variance with what was reported previously (Ling et al., 2003), studies with purified peptides, including the same peptides used by Ling et al. (2003), did not reveal an effect of Y649 phosphorylation on the interaction of PIPKIγ90 with talin. Furthermore, structural data indicate that phosphorylation of Y649 would be unlikely to directly perturb the interaction between these two proteins (Liddington et al., 2003; de Pereda et al., 2004). In contrast, the negative effect of S650 phosphorylation on the interaction of PIPKIγ90 with talin is consistent with structural predictions pointing to an intimate interaction of S650 with talin (Liddington et al., 2003) and with the mutagenesis experiments reported here.

However, we did find that the presence of phosphate on Y649 strongly inhibits the phosphorylation of S650, and vice versa. Thus, Src-dependent phosphorylation of Y649 may act indirectly to enhance talin binding by inhibiting phosphorylation of S650. Conversely, in addition to directly blocking the interaction with talin, phosphorylation of S650, in a feed-forward mechanism, blocks the phosphorylation reaction at Y649 that inhibits S650 phosphorylation. Src family kinases have been implicated both in synaptic function and in focal adhesion dynamics (Purcell and Carew, 2003; Carragher and Frame, 2004), thus supporting a physiological significance of this bidirectional control of PIPKIγ90.

While our manuscript was in review, a study by de Pereda et al. (2004) reported a crystallographic analysis of the interaction between the F3 subdomain of talin and the COOH-terminal tail of mouse PIPKIγ. The structure is consistent with the results of our phosphorylation studies. It predicts that phosphorylation of serine 650 of human PIPKIγ90 would block the interaction, and explains the lack of effect that we report here for the phosphorylation of tyrosine 649. Dissociation constant values determined by ITC for the talin–PIPKIγ90 peptide interaction in this new study (see Fig. S1 in de Pereda et al., 2004) are in the same range of those reported by us and show only a minor (1.6-fold) positive effect of phosphorylation of tyrosine 644, which corresponds to human tyrosine 649, on the interaction (de Pereda et al., 2004). Overall, these new findings are in very good agreement with our results.

Materials and methods

Antibodies

Polyclonal antibodies against human PIPKIγ90 and GST were prepared as described previously (McPherson et al., 1994; Di Paolo et al., 2002). A polyclonal anti-GFP antibody was a gift from Susan Ferro-Novick (Yale University, New Haven, CT). For the pS650-specific antibody, we immunized a rabbit with a synthetic phosphopeptide corresponding to residues 646–654 (SWVYpSPLHYC) of human PIPKIγ90. The COOH-terminal cysteine residue was added to the peptide for affinity purification using Sulfolink gel (Pierce Chemical Co.). The following antibodies were obtained from commercial sources; rat mAb to HA tag (Roche); mouse mAb to Cdk5 and amphiphysin 2 (Upstate Biotechnology), to talin and tubulin (Sigma-Aldrich) and to phospho-MAPK1/2 (New England Biolabs); rabbit polyclonal Ab to MAPK1/2 (New England Biolabs, Inc.) and to phospho-histone H3 (Upstate Biotechnology).

DNA constructs and protein expression

Constructs of human PIPKIγ87 (640 aa), PIPKIγ90 (668 aa) and 28–aa tail (641–668) of PIPKIγ90, and the F3 subdomain and head domain of talin were subcloned into pcDNA3-HA, pGEX-6P-1, or pEGFP-C2 vectors, as described previously (Di Paolo et al., 2002; Krauss et al., 2003). We also subcloned PIPKIγ87 and PIPKIγ90 into a modified version of pET15 vector (Novagen) with an NH_2-terminal His_6-tag. S650 in the PIPKIγ90 constructs was mutated to alanine, aspartic acid, or asparagine by a QuikChange site-directed mutagenesis kit (Promega), and the mutations were confirmed by DNA sequencing. Full-length GST- and His_6-PIPKIγ90 fusion proteins expressed in Sf9 cells and *Escherichia coli* strain BL21, respectively, were purified using glutathione-Sepharose beads (Amersham Biosciences) and Talon metal affinity resin (BD Biosciences) following the manufacturer's protocols and dialyzed against TBS. Expression constructs encoding p35, Cdk5, and mut-Cdk5 in the pCMV vector were gifts from L.-H. Tsai (Harvard Medical School, Boston, MA). A construct of GST-integrin β_1 tail was a gift from J. Ylanne (University of Oulu, Oulu, Finland).

In vitro phosphorylation

p35–Cdk5 and cyclin B1–Cdk1 complexes, expressed and purified from Sf9 cells coinfected with baculovirus encoding each component, were gifts from Y. Kim and P. Greengard (The Rockefeller University, New York, NY) and Y. Wang and G. Warren (Yale University), respectively. Purified GST-PIPKIγ90 or His_6-PIPKγ87/90 fusion proteins (200–300 ng) were incubated with p35–Cdk5 complex (Lee et al., 2004) or cyclin B1–Cdk1 complex (Wang et al., 2003) at an ~1:1 stoichiometric ratio in the presence of 10 μCi γ-[^{32}P]ATP (1 Ci = 37 GBq) for up to 30 min at 30°C. PIPKIγ90 phosphorylation by MAPK1 (Upstate Biotechnology) was performed according to the manufacturer's protocol. Kinase reactions (30–40 μl final volume) were stopped by the addition of SDS-PAGE sample buffer, and samples were analyzed by SDS-PAGE and autoradiography. In case of peptide phosphorylation by c-Src (Upstate Biotechnology) and p35/Cdk5, each kinase was mixed with the 12-mer peptides (each 25 μM) in the presence of 400 μM ATP and 40 μCi γ-[^{32}P]ATP. c-Src phosphorylation was performed in 25 mM Tris-HCl, pH 7.2, 30 mM $MgCl_2$, 0.5 mM EGTA, and 62.5 μM Na_3VO_4, and p35/Cdk5 phosphorylation was assayed as described above. After a 20-min incubation at 30°C, reaction mixtures (40 μl) were stopped by adding 20 μl of 40% TCA solution, and a portion of reaction mixtures (25 μl) was spotted onto P81 phosphocellulose paper (Whatman). All reactions were in the linear range and consumed <1% of peptide substrates. After washing five times with 0.75% phosphoric acid and once with acetone, radiolabeling of the peptides was quantified by Cerenkov counting.

Identification of phosphorylation sites and 2-D peptide mapping

12 μg His_6-PIPKγ90 labeled with γ-[^{32}P]ATP by p35–Cdk5, as described in the previous paragraph, was excised from SDS-PAGE and digested with trypsin (Lee et al., 2004). The resulting peptides were separated by HPLC and detected by Cerenkov counting. Peptide masses containing a phosphate group were determined by MALDI/MS, and the phosphorylation sites were confirmed by further radio sequencing of the peptides. 2-D phosphopeptide mapping was performed as previously described (Hsieh-Wilson et al., 2003). In brief, after centrifugation of the trypsin-digested radiolabeled material, supernatants were collected and lyophilized. Pellets were then washed with distilled water and running buffer (10% acetic acid and 1% pyridine in water) several times. Lyophilized pellets were dissolved in running buffer and spotted onto cellulose TLC plates. The plates were sequentially subjected to electrophoresis in the running buffer, pH 3.5, at 400 V, and then to chromatography in a buffer (25% 1-butanol, 7.5% acetic acid, and 37.5% pyridine in water). After drying the plates, autoradiography was performed.

In vitro talin-binding assay and ITC

The talin overlay assay was performed as described previously (McPherson et al., 1994). 100–200 ng of purified His_6-PIPKIγ90 fusion proteins transferred onto nitrocellulose membranes were incubated without or with GST-F3 fusion protein (2 μg/ml). Membranes were blotted with anti-GST antibody. For the pull-down assay from rat brain extracts, His_6-PIPKIγ90 fusion proteins (4–5 μg) coupled to Talon metal affinity beads in TBS containing 1% (vol/vol) Triton X-100 were mixed with rat brain extracts (6.4 mg) prepared in buffer A (20 mM Hepes, pH 7.4, 120 mM KCl, 1 mM DTT, 1 mM EDTA, 1 mM EGTA, 1 mM Na_3VO_4, 1 mM PMSF, and 1% [vol/vol] Triton X-100) for 3 h at 4°C. ITC measurements were performed using a Microcal VP-ITC isothermal titration calorimeter equipped with a PC running VPViewer software. Dissociation constant values were obtained from the data collected automatically and analyzed with Origin software. In each experiment, 3-μl aliquots of peptide solution (1 mM) were injected into a calorimetric cell preloaded with 1.4267 ml GST-talin head domain (16.4 μM) using a rotating stirrer syringe (250-μl vol) every

250 s at 37°C. All solutions included 20 mM sodium phosphate, pH 7.4, and 150 mM NaCl and were degassed. To estimate a blank heat effect associated with dilution and mechanical phenomena, peptide injections were performed after saturation of binding.

Cell culture, transfection, and immunoprecipitation

CHO cells were grown in DMEM supplemented with 10% FBS and antibiotics and transfected for 24 h with expression constructs of WT or mutants of HA-PIPKIγ90 and of GFP-PIPKIγ90, p35, Cdk5 or mut-Cdk5 using Lipofectamine 2000 (Life Technologies) according to the instruction manual. For immunoprecipitation of PIPKIγ90 and talin, the cell lysates prepared in the buffer A were incubated with anti-PIPKIγ90 and antitalin antibodies for 3 h at 4°C, and then were incubated with protein A– and protein G–Sepharose beads (Amersham Biosciences) for 2 h, respectively. Cell lysates and bound materials washed with the buffer A five times were analyzed by SDS-PAGE and Western blotting. A similar protocol was used for the affinity chromatography of talin from transfected CHO cells on the GST-integrin β_1 tail, except that glutathione Sepharose beads were used.

Synaptosomes

Rat brain synaptosomes were prepared as described previously (Bauerfeind et al., 1997; Lee et al., 2004). In brief, 200-μl aliquots of synaptosomes were preincubated in the "control buffer" with or without Ca^{2+} for 20 min at 37°C, and then diluted with an equal volume of either control buffer or stimulation buffer containing high K^+. When inhibitors of protein kinases and phosphatases were used, the inhibitors were present during both the preincubation and the stimulation steps. For analysis of total phosphorylation of PIPKIγ90, synaptosomes were labeled with 2 mCi/ml γ-[^{32}P]orthophosphate for 1 h prior to the preincubation. After a 1-min stimulation, 100 μl of buffer B (20 mM Hepes, pH 7.4, 50 mM NaCl, 50 mM Na_3PO_4, 50 mM NaF, 5 mM EDTA, 5 mM EGTA, 5 mM β-glycerophosphate, 1 mM PMSF, and 1% Triton X-100 [vol/vol]) supplemented with 10% SDS (final 2% SDS) was directly added to synaptosomes for lysis and incubation mixtures were boiled for 10 min. For repolarization, synaptosomes stimulated by high K^+ were rapidly harvested, resuspended in control buffer, and were further incubated for 15 min before lysis. For PIPKIγ90 immunoprecipitation, synaptosomal lysates were diluted with 9 vol of the buffer B and added to protein A–Sepharose beads precoated with anti-PIPKIγ90 antibody for 2 h at 4°C. Western blotting or autoradiography of synaptosomal lysates and PIPKIγ90 immunoprecipitates were performed after SDS-PAGE.

Mitotic synchronization

U87MG cells were grown in minimum essential medium with Earle's salts supplemented with 10% FBS, 0.1 mM nonessential amino acids, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.15% sodium bicarbonate, and antibiotics. To prepare both mitotic and G_1 interphase cells, subconfluent U87MG cells were treated with 250 ng/ml nocodazole (Sigma-Aldrich) for 18 h (Chen et al., 1999). Mitotic cells released into culture medium were collected by mechanical shake-off and washed with PBS. To harvest G_1 interphase cells, the mitotic cells were replated into fresh culture medium and further incubated for 10 h. CHO cells transfected with HA-PIPKIγ90 constructs were processed as described above. Preparation of cell lysates in the buffer A, PIPKIγ90 immunoprecipitation, SDS-PAGE, and Western blotting were performed as described above.

Immunofluorescence

Immunofluorescence of transfected NIH3T3 cells was performed as described previously (Di Paolo et al., 2002). Fluorescence was visualized with a microscope (Axioplan 2; Carl Zeiss MicroImaging, Inc.) equipped with a cooled CCD camera (Orca ER2; Hamamatsu) using a Plan-Apochromat (63×, 1.4 NA) oil immersion objective at room temperature. Images were acquired with Metamorph software (Universal Imaging) and processed using Adobe Photoshop.

Miscellaneous

Amounts of proteins were calculated by a BCA assay kit (Pierce). SDS-PAGE and Western blotting were conducted using standard procedures.

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