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Serine and Threonine Phosphorylation of the Paxillin LIM Domains Regulates Paxillin Focal Adhesion Localization and Cell Adhesion to Fibronectin

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We have previously shown that the LIM domains of paxillin operate as the focal adhesion (FA)-targeting motif of this protein. In the current study, we have identified the capacity of paxillin LIM2 and LIM3 to serve as binding sites for, and substrates of serine/threonine kinases. The activities of the LIM2- and LIM3-associated kinases were stimulated after adhesion of CHO.K1 cells to fibronectin; consequently, a role for LIM domain phosphorylation in regulating the subcellular localization of paxillin after adhesion to fibronectin was investigated. An avian paxillin-CHO.K1 model system was used to explore the role of paxillin phosphorylation in paxillin localization to FAs. We found that mutations of paxillin that mimicked LIM domain phosphorylation accelerated fibronectin-induced localization of paxillin to focal contacts. Further, blocking phosphorylation of the LIM domains reduced cell adhesion to fibronectin, whereas constitutive LIM domain phosphorylation significantly increased the capacity of cells to adhere to fibronectin. The potentiation of FA targeting and cell adhesion to fibronectin was specific to LIM domain phosphorylation as mutation of the amino-terminal tyrosine and serine residues of paxillin that are phosphorylated in response to fibronectin adhesion had no effect on the rate of FA localization or cell adhesion. This represents the first demonstration of the regulation of protein localization through LIM domain phosphorylation and suggests a novel mechanism of regulating LIM domain function. Additionally, these results provide the first evidence that paxillin contributes to "inside-out" integrin-mediated signal transduction.

INTRODUCTION

Paxillin is a 68-kDa focal adhesion (FA)[1] phosphoprotein that is localized to actin–membrane attachment sites in vivo. A well-documented substrate of protein kinases, paxillin is tyrosine phosphorylated to a high stoichiometry (20–30%) during various cellular events associated with cell adhesion, remodeling of the actin-based cytoskeleton, and growth control (reviewed by Turner, 1994). The phosphorylation state of paxillin has been shown to be dynamically regulated in several cell types by physiologic stimuli including bombesin, PDGF, nerve growth factor, and angiotensin II (Zachary *et al.*, 1993; Rankin and Rozengurt, 1994; Melamed *et al.*, 1995; Turner *et al.*, 1995). In addition, it is one of approximately five proteins phosphorylated to a high level on tyrosine residues in a developmentally regulated manner in the chick embryo (Turner, 1991). Concomitant with paxillin tyrosine phosphorylation during these events is the activation of the tyrosine kinase, focal adhesion kinase (FAK) (Burridge *et al.*, 1992; Turner *et al.*, 1993, 1995). Recently, the FAK-binding sites, as well as the predominant targets of tyrosine phosphorylation of paxillin by FAK (Y31 and Y118), were identified on the amino terminus of paxillin (Turner and Miller, 1994; Bellis *et al.*, 1995;

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[1] Abbreviations used: CRS, cytoplasmic retention signal; FA, focal adhesion; FAK, focal adhesion kinase; PAA, phosphoamino acid analysis.

Schaller and Parsons, 1995; Brown *et al.*, 1996). After phosphorylation, these tyrosine residues function as SH2-binding sites for the adaptor protein crk, a protein involved in modulating Ras family signal transduction (Schaller and Parsons, 1995).

Unlike tyrosine phosphorylation of paxillin, the capacity of paxillin to serve as a substrate for serine/threonine kinases has not been examined in great detail. Paxillin migrates as a diffuse band of ~68 kDa and, on the basis of comparison of phosphotyrosine and paxillin Western blotting, it has been suggested that serine/threonine phosphorylation contributes to the reduced mobility isoforms (Turner *et al.*, 1990; Zachary *et al.*, 1993). Indeed, two recent reports detailed substantial phosphorylation of paxillin on serine in response to cell adhesion to vitronectin (De Nichilo and Yamada, 1996) and fibronectin (Bellis *et al.*, 1997). Additionally, phosphoamino acid analysis (PAA) of in vivo ^{32}P-labeled paxillin revealed phosphoserine and phosphothreonine as well as phosphotyrosine (Salgia *et al.*, 1995; De Nichilo and Yamada, 1996; Bellis *et al.*, 1997; Vande Pol *et al.*, 1998). Examination of the primary amino acid sequence of paxillin reveals multiple serine/threonine phosphorylation sites as well as many other motifs that have been implicated in protein–protein interactions including SH2- and SH3-binding domains, five LD repeats, and four LIM double-zinc finger domains (Turner and Miller, 1994; Brown *et al.*, 1996).

The LIM domains of paxillin are cysteine/histidine-rich double zinc fingers of 50 amino acids that are related to a group of LIM-containing, cytoskeleton-associated proteins that includes zyxin, cysteine-rich protein, and muscle LIM protein (Sánchez-García and Rabbitts, 1994; Gill, 1995). LIM domains function in protein–protein interactions (Gill, 1995) and, in this regard, they represent the principal determinant of paxillin localization to FAs (Brown *et al.*, 1996) and muscle LIM protein and cysteine-rich protein localization to the nucleus and along actin filaments (Arber and Caroni, 1996).

Although the phosphorylation state of paxillin is closely tied to events involving modulation of FAs and the actin cytoskeleton, it is as yet unclear what role these posttranslational modifications play in mediating paxillin function. By identifying sites on paxillin involved in protein–protein interactions and examining the contribution of these components to cellular function, we aim to define paxillin activity. Paxillin LIM domain fusion proteins were generated and used to affinity isolate proteins that associate with paxillin through the FA-targeting portion of the molecule. We identified the specific binding of serine/threonine protein kinase(s) to paxillin LIM2 and LIM3. A role for paxillin phosphorylation in FA localization and cellular adhesion was examined by transfection of CHO.K1 with mutated paxillin molecules. LIM domain phosphorylation was found to temporally regulate the availability of paxillin to integrate into FAs upon adhesion to fibronectin. Additionally, these phosphorylated residues potentiated the ability of cells to adhere to fibronectin, suggesting a novel function for paxillin in "inside-out" signaling.

MATERIALS AND METHODS

GST-Paxillin Fusion Proteins

Glutathione-*S*-transferase (GST), and GST-paxillin LIM1, LIM2, and LIM2 mutant molecules as well as GST-LIM4 were produced and purified as previously described (Brown *et al.*, 1996). GST-LIM3 and LIM3 mutant molecules were generated as follows. *Escherichia coli* (DH5α) transformed with the appropriate pGEX2T-LIM3 paxillin construct was grown overnight, diluted 1:30 into a hyperosmotic Luria-Bertani medium (10 g/l yeast extract, 10 g/l Bacto-tryptone, 0.5 g/l NaCl, 1 M Sorbitol, and 2.5 mM Betaine) and grown for an additional 5 h. Protein expression was induced for 12 h at ambient temperature by the addition of 0.01 mM isopropyl-β-D-thiogalactopyranose. Cells were harvested by centrifugation at 10,000 $\times$ *g* for 10 min. Fusion protein was purified by lysis of bacteria in Tris-buffered saline, pH 8.0, containing 2 mg/ml lysozyme, 0.1% β-mercaptoethanol, 1% Triton X-100, and a mixture of protease inhibitors (Complete, Boehringer Mannheim, Indianapolis, IN) for 30 min at room temperature. Bacterial cell wall debris was removed by centrifugation at 12,000 $\times$ *g* for 15 min with the fusion protein recovered from the supernatant by incubation with glutathione-Sepharose 4B (Pharmacia, Piscataway, NJ) according to the manufacturers' instructions.

GST-Paxillin Precipitation Kinase Assays

For kinase assays, a lysate of embryonic chicken gizzard or cultured cells was prepared by homogenizing the tissue/cells in 10 volumes (wt/vol) of lysis buffer containing 50 mM Tris-HCl, pH 7.6, 50 mM NaCl, 1 mM EGTA, 2 mM $MgCl_2$, 0.1% mercaptoethanol, 1% Triton X-100, and a mixture of protease inhibitors (Complete). The lysate was clarified at 14,500 $\times$ *g* for 15 min at 4°C. Aliquots of tissue/cell lysate (1 mg of tissue lysate, 250 μg cell lysate) were incubated with 5 μg of the various GST-paxillin fusion proteins coupled to the glutathione-Sepharose 4B beads or with GST-glutathione-Sepharose 4B for 90 min at 4°C and washed extensively in lysis buffer, followed by washing with 1 ml kinase buffer (10 mM HEPES, pH 7.5, 3 mM $MnCl_2$). The kinase buffer was aspirated and the pellet resuspended in 20 μl kinase buffer containing 10 μCi of [^{32}P]-γ-ATP. The phosphorylation reaction proceeded at room temperature for 20 min and was terminated by boiling directly in 2$\times$ SDS-PAGE sample buffer. The samples were processed by SDS-PAGE, stained with Coomassie blue to confirm equal fusion protein loading, dried, and analyzed by autoradiography. PAA was completed according to standard procedures (van der Geer and Hunter, 1994).

Cell Culture, Transfection, and Immunofluorescence

CHO.K1 cells were cultured in modified Ham's F-12 (Mediatech, Washington, D.C.) supplemented with 10% (vol/vol) heat-inactivated, certified FBS (Life Technologies, Grand Island, NY) and 1% penicillin-streptomycin (complete medium) at 37°C in a humidified chamber with 5% CO_2. Production of pcDNA3-paxillin vectors, generation of site-directed mutagenesis products, and transfection of CHO.K1 cells using LipoFECTAMINE (Life Technologies) were as described elsewhere (Brown *et al.*, 1996).

To generate CHO.K1 clones, heterogeneous populations of CHO.K1 cells stably transfected with pcDNA3 vectors encoding avian paxillin proteins were diluted into complete Ham's F-12 containing 2 mg/ml G418 (Mediatech), to select cells containing the

pcDNA3 vector, and monitored for formation of individual, well-spaced colonies. The cellulose disk method of cloning was utilized (Domann and Martinez, 1995) with clones examined and chosen for homogenous expression of the respective avian paxillin molecule as well as relative level of expression. CHO.K1 clones were maintained in complete Ham's F-12 with 250 μg/ml G418.

Indirect immunofluorescence analyses were performed by plating a heterogeneous population of avian paxillin-expressing CHO.K1 cells onto glass coverslips. After 24 h in complete medium, the cells were fixed for 8 min with 3.7% formaldehyde in PBS, washed for 10 min with Tris-buffered saline (TBS), permeablized for 2 min in 0.2% Triton X-100 in TBS, followed by washing for 10 min in TBS. An avian-specific paxillin polyclonal antibody (Pax1) was used for detection of ectopically expressed paxillin, and the phosphotyrosine-specific monoclonal antibody PY20 was utilized to identify FAs. "Efficiency of localization" describes the ability of the ectopic avian paxillin to effectively localize to a site of FA. It is measured by comparing the size, intensity, and overlap of the indirect immunofluorescence avian paxillin signal relative to that of the PY20 double-label.

Rate of FA Localization

For the time course of avian paxillin localization to FA studies, heterogeneous, stably transfected CHO.K1 populations were placed in suspension by brief treatment with PBS–1 mM EDTA followed by washing in serum-free Ham's F-12 and resuspension in serum-free Ham's F-12 at a concentration of 1×10^5 cells/ml. The cells were maintained in suspension with gentle rocking at 37°C in a humidified chamber with 5% CO_2. After 1 h, 1×10^5 cells were plated in serum-free medium into a 60-mm dish containing fibronectin-coated coverslips (10 μg/ml; human plasma fibronectin, Sigma Chemical, St. Louis, MO). At the designated time after plating, the coverslips were processed, as above, i.e., double-labeled for indirect immunofluorescence microscopy using Pax1 and either PY20 to colabel FAs or rhodamine phalloidin to label stress fibers.

For each time point, a total of 150–200 cells were counted, and the localization of avian paxillin to FAs within the transfected cells was assessed. At each time point, all cells had formed FAs as detected with the PY20 monoclonal antibody. The number of avian paxillin transfectants displaying FA localization of avian paxillin (at least 4 focal contacts per cell) was divided by the total number of transfectants present within the counted fields. This calculation was taken as a measure of the rate of avian paxillin molecule localization to FAs and described as "Percent localization to FA." The identity of the transfected construct was not known until after the data were analyzed. All experiments were performed in duplicate with at least three independent experiments executed. These data were then analyzed by Student's *t* test using Microsoft Excel 5.0.

Cell Adhesion Assays

Cell adhesion assays were performed by precoating 96-well dishes with 10 μg/ml fibronectin or 3% BSA for 3 h at 37°C followed by washing in Earle's Balanced Salt Solution and blocking with 3% BSA in serum-free Ham's F-12 overnight at 37°C. CHO.K1 clones expressing the various avian paxillin mutant molecules were processed as in the localization studies above. Two independent clones of each avian paxillin mutant molecule were utilized to eliminate clone-specific effects on cell adhesion. CHO.K1 cells were resuspended in Ham's F-12 containing 1% BSA, and 5×10^4 cells in a volume of 50 μl were transferred to each well after which the 96-well plates were incubated for 30 min at 37°C. The plates were then washed three times with 150 μl Earle's Balanced Salt Solution while the 96-well plate was subjected to constant agitation for 10 s at vortex setting 3 in a 96-well platform adaptor on a Vortex Genie II apparatus for each wash. The cells were then washed once with phenol red-free Ham's F-12 containing 10% FBS and then incubated for 4 h with phenol red-free Ham's F-12 containing 10% FBS and 0.5 mg/ml 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT). The plates were washed once with phenol red-free Ham's F-12 and the precipitate was processed according to standard procedures. Aliquots of the cells used in the adhesion assays were examined in parallel by immunofluorescence using the avian-specific Pax1 antibody to verify expression. All experiments were performed in duplicate (16 wells per experiment) with at least three independent experiments executed. These data were then analyzed by Student's *t*-test using Microsoft Excel 5.0.

RESULTS

Phosphorylation of the LIM domains of Paxillin on Threonine and Serine

Precipitation kinase assays have been used successfully to identify the association of paxillin with FAK (Turner and Miller, 1994; Bellis *et al.*, 1995) and a serine kinase (Bellis *et al.*, 1997). Therefore, the capacity of the LIM domains of paxillin to operate as binding sites for and substrates of protein kinases was examined. The individual LIM domains of paxillin were produced as GST fusion proteins (for schematic representation, see Figure 1) and bound to glutathione-Sepharose 4B beads to serve as a solid support in the affinity isolation of paxillin LIM domain protein kinases. These fusion proteins were incubated with chicken smooth muscle lysate, and the resulting complex was subjected to in vitro kinase assay. The phosphorylated fusion proteins are shown in Figure 2A. GST-LIM2 and GST-LIM3 fusion proteins were heavily phosphorylated whereas no phosphorylation of GST, GST-LIM1, or GST-LIM4 was evident. Thus, paxillin LIM2 and LIM3 are capable of specifically binding protein kinases. Thrombin cleavage of the phosphorylated GST-LIM fusion proteins confirmed that phosphorylation was restricted to the respective LIM domain (our unpublished observations).

Expression of the LIM-associated kinases was found to be widespread. In addition to smooth muscle tissue, LIM2 and LIM3 protein kinase activity was present in skeletal and cardiac muscle as well as brain, liver, lung, chicken embryo fibroblasts, and the CHO.K1 and NIH3T3 cell lines (our unpublished observations). PAA revealed that the phosphorylation of GST-LIM2 was restricted to threonine, whereas GST-LIM3 was phosphorylated on serine (Figure 2C).

Mutation Analysis of Paxillin LIM Domain Phosphorylation

To identify those residues that participate in LIM2-kinase association and phosphorylation, several mutations were generated (Figure 3A). The two separate threonine residues, which reside on the first zinc finger, were mutated to valine to determine the principal site of threonine phosphorylation on LIM2. Mutation of threonine 398 ($LIM2^{T398V}$) showed only a slight reduction in phosphorylation (lane 6) relative to the mutation $LIM2^{C411A}$ that exhibited phosphorylation

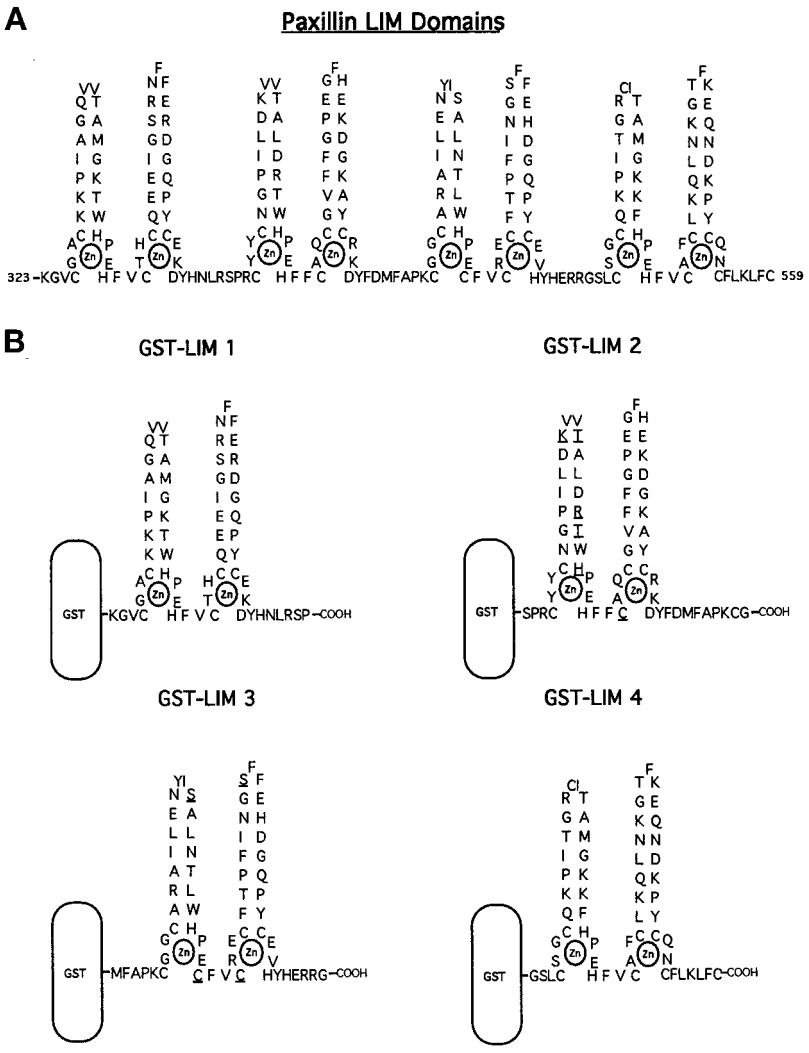

Figure 1. A schematic representation of the four LIM domains of paxillin. (A) The four LIM domains of paxillin are found at the carboxyl terminus of the protein and span amino acids 323–559. Each LIM motif is composed of two zinc fingers. (B) Detail of the amino acid residues comprising the individual LIM domain-GST fusion proteins. The residues underlined were targets for mutagenesis in this study.

levels equivalent to wild type (lane 2). In contrast, mutation of threonine 403 (LIM2^{T403V}) resulted in the elimination of phosphorylation (lane 7). LIM2^{T403} does not clearly fall within any known consensus sequences and may define a novel site of protein phosphorylation (Pearson and Kemp, 1991; Songyang *et al*., 1994, 1996).

Binding of zinc is essential for the proper folding of LIM domains (Gill, 1995); thus, a series of mutations were constructed targeting the zinc-chelating amino acid residues of the LIM domain. Elimination of one of the four zinc-chelating residues disrupts zinc binding and therefore perturbs the structure of the zinc finger (Feurerstein *et al*., 1994; Schmeichel and Beckerle, 1997). Disruption of the first finger of LIM2 by mutagenesis of histidine 405 to isoleucine (LIM2^{H405I}) resulted in a complete elimination of phosphorylation (Figure 3A, lane 1) whereas two separate mutations in the second finger (LIM2^{C411A} or LIM2^{C432A}) were without a significant effect (lanes 2 and 3), establishing that a structurally intact first zinc finger component of the LIM2 domain was necessary and sufficient for the functional recruitment of the protein kinase.

Many protein kinases have a requirement for flanking basic residues in the recognition and binding of substrates (Pearson and Kemp, 1991; Songyang *et al*., 1994, 1996). Consequently, the role of lysine 395 and arginine 402 was tested by generating mutations of these residues. Precipitation kinase assay using either of these mutated fusion proteins revealed a considerable reduction in the phosphorylation of LIM2 (Figure 3A, lanes 4 and 5).

Similar experiments were performed to determine the site of phosphorylation of LIM3; Figure 3B). Mutagenesis of either of the two serine residues to alanine that are present on LIM3, amino acids 457 (LIM3^{S457A}) and 481 (LIM3^{S481A}), did not reduce phosphorylation of LIM3 (Figure 3B, lanes 3 and 4), indicating that both

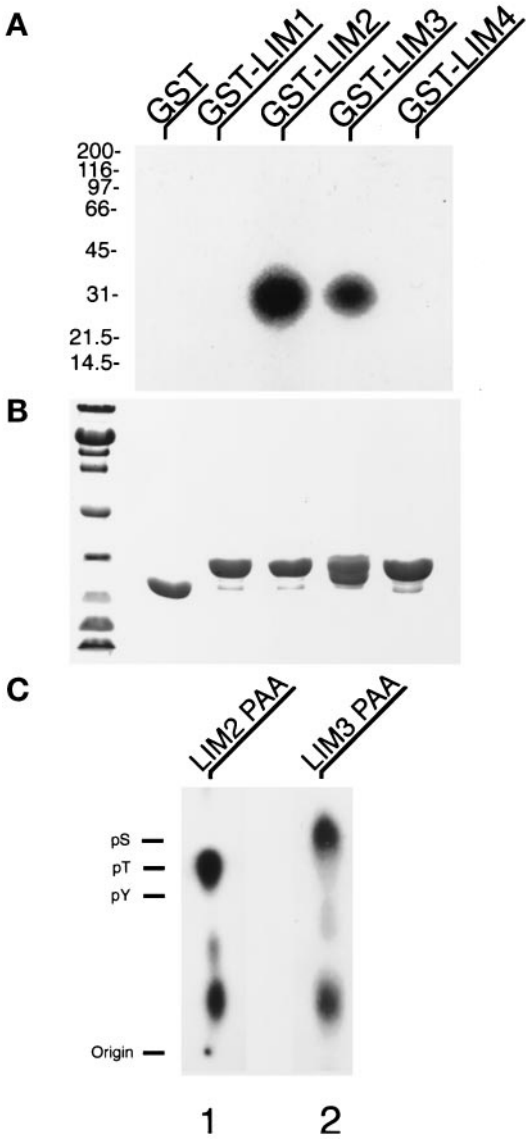

Figure 2. Phosphorylation of paxillin LIM domains 2 and 3 on threonine and serine. (A) GST fusion proteins comprising the four individual LIM domains of paxillin were incubated with avian smooth muscle lysates and washed free of unbound protein, followed by protein kinase assay as described in MATERIALS AND METHODS. Lane 1, GST alone; lane 2, GST-LIM1; lane 3, GST-LIM2; lane 4, GST-LIM3; and lane 5, GST-LIM4. Only GST-LIM2 and GST-LIM3 specifically precipitated and were phosphorylated by protein kinases. (B) Coomassie brilliant blue staining of the GST precipitation kinase assay SDS-polyacrylamide gel to show equivalent loading of the fusion proteins. (C) PAA was performed on the phosphorylated GST-LIM2 and GST-LIM3 fusion proteins. Comigration of ninhydrin-stained phosphoamino acid standards revealed that the phosphorylation of LIM2 was restricted to the amino acid threonine and paxillin LIM3 on serine. Lower spots are partial hydrolysis products.

residues function as substrates of the serine kinase. Attempts to produce stable and soluble LIM3$^{S457A/S481A}$ double-serine mutant fusion protein for examination

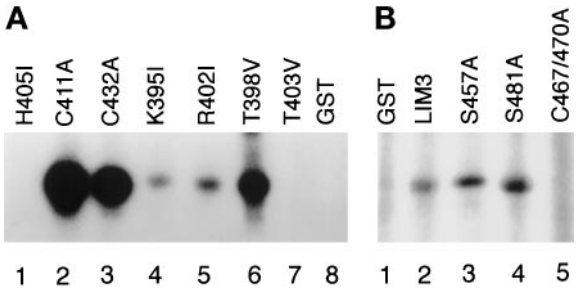

Figure 3. Paxillin LIM domain–kinase association is LIM domain structure-dependent. (A) GST-LIM2 fusion proteins were generated containing point mutations of residues potentially involved in kinase binding or phosphorylation and subjected to in vitro kinase assay as in MATERIALS AND METHODS. Lane 1, amino-terminal zinc finger, zinc-chelating histidine 405 to isoleucine; lane 2, carboxyl-terminal zinc finger, zinc-chelating cysteine 411 to alanine; lane 3 carboxyl-terminal zinc finger, zinc-chelating cysteine 432 to alanine; lane 4, lysine 395 to isoleucine; lane 5, arginine 402 to isoleucine; lane 6, threonine 398 to valine; lane 7, threonine 403 to valine; lane 8, GST only. Phosphorylation of C411A was equivalent to wild-type LIM2. GST-LIM3 fusion proteins were generated containing point mutations of residues potentially involved in kinase binding or phosphorylation. Lane 1, GST; lane 2, serine 457 to alanine; lane 3, serine 481 to alanine; lane 4, amino- and carboxyl-terminal zinc finger, zinc-chelating cysteines 467 and 470 to alanine.

in precipitation kinase assays were not successful. To determine whether the zinc fingers of LIM3 were important in kinase binding or phosphorylation, a double-point mutant was constructed in which the zinc-chelating amino acids cysteine 467 (LIM3^{C467A}) and cysteine 470 (LIM3^{C470A}) were mutated to alanine. Elimination of the structural integrity of the LIM3 zinc fingers by site-directed mutagenesis resulted in a complete elimination of phosphorylation (lane 5).

Phosphorylation of the LIM Domains of Paxillin in CHO.K1 Fibroblasts

To address the in vivo relevance of LIM phosphorylation during adhesion we measured kinase activity in lysates from CHO.K1 cells maintained in suspension and after adhesion to fibronectin (Figure 4). Cells held in suspension had considerable basal LIM2 kinase activity while LIM3 kinase activity was not detectable (lane 2 and 3, respectively). When cells were plated on fibronectin for 10 min, an induction of LIM3 phosphorylation was evident (compare lanes 3 and 6) and remained elevated for up to 120 min (lane 12). At 120 min after plating, a slight stimulation of LIM2 phosphorylation also was observed (compare lanes 2 and 11). Thus, the activities of serine/threonine protein kinases capable of phosphorylating paxillin LIM2 and LIM3 were modulated in response to changes in cellular adhesion states.

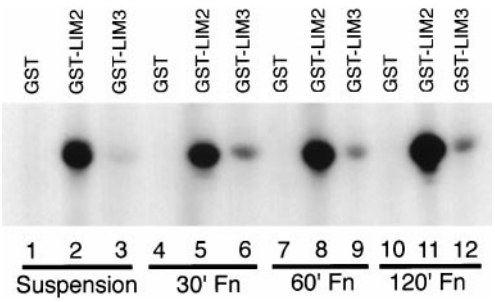

Figure 4. Stimulation of LIM2 and LIM3 serine/threonine kinases after adhesion to fibronectin. GST (lanes 1, 4, 7, and 10), GST-LIM2 (lanes 2, 5, 8, and 11), or GST-LIM3 (lanes 3, 6, 9, and 12) fusion proteins were incubated with CHO.K1 lysates, washed free of unbound protein, followed by in vitro kinase assay as in MATERIALS AND METHODS. Lanes 1–3, Lysates from cells maintained in suspension for 60 min; lanes 4–6, cells plated on 10 μg/ml fibronectin-coated Petri dishes for 30 min; lanes 7–9, cells plated for 60 min; lanes 10–12, cells plated for 120 min before harvesting for preparation of lysates for use in in vitro kinase assays. Laser scanning densitometry of the phosphorylated GST-LIM fusion protein autoradiograph signal revealed that CHO.K1 adhesion to fibronectin for 30 min stimulated LIM3 kinase activity 4.5-fold over cells maintained in suspension. After this peak stimulation, the levels decreased over time, to 3.5-fold by 120 min. LIM2 phosphorylation gradually increased 1.2-fold over suspension levels by 120 min.

Contribution of Phosphorylation to Paxillin FA Localization

Previously we determined that a structurally intact LIM3 motif is absolutely required, and a structurally intact LIM2 motif necessary, for optimal targeting of paxillin to FAs (Brown *et al*., 1996). To determine whether phosphorylation of LIM2 or LIM3 contributed to targeting of paxillin to FAs, we produced paxillin constructs containing site-directed mutations of the potential phosphorylation sites within LIM2 and LIM3. After transfection into CHO.K1 fibroblasts, the effect on FA targeting of the exogenous avian paxillin was examined at steady state (Figures 5 and 6).

Avian paxillin LIM2^{T403V} mutant molecules localized to FA, similar to that of wild-type avian paxillin (Figure 5, compare panel C with panel A). A threonine-to-glutamic acid point mutation (LIM2^{T403E}), designed to mimic threonine phosphorylation, was produced to further examine a role for phosphorylation in FA targeting. LIM2^{T403E} localized to FAs and showed some localization along stress fibers (Figure 5E).

In contrast to the LIM2^{T403V} mutant molecule, paxillin containing LIM3 serine mutations (LIM3^{S457A}, LIM3^{S457D}, LIM3^{S481A}) targeted to FAs much less efficiently than wild-type avian paxillin (Figure 6, compare panels A, C, and E with Figure 5A). However, the phospho-mimetic LIM3^{S481D} molecule demonstrated localization comparable to wild-type avian paxillin (Figure 6G). The reduced FA localization observed with some of the LIM mutants was not due to a disruptive effect on stress fiber formation. Rhodamine-phalloidin double labeling revealed an actin network indistinguishable from wild type (data not shown). In addition, immunostaining of these FAs using PY20 revealed no significant reduction in FA number or intensity of staining.

Effect of Phosphorylation Mutations on Localization after Plating on Fibronectin

The above data demonstrated that mutations of phosphorylatable residues within the LIM domains affect the overall efficiency of paxillin localization to FA that have reached a steady state. However, at this stage of adhesion the presence of an endogenous pool of wild-type paxillin may mask effects of these mutations on the formation of FAs.

To evaluate the effect of these mutations on the targeting of paxillin to nascent FAs, the localization of avian paxillin to FAs was examined at various times after plating on fibronectin. The percentage of transfected cells that demonstrated FA targeting of the ectopically expressed paxillin protein at each time point was determined.

The rate of localization of proteins containing mutations in the amino-terminal half of the protein were examined first. Compared with wild-type avian paxillin, molecules containing mutations of Y31/118 or S188/190, the four sites known to be phosphorylated in response to adhesion, demonstrated no difference in the rate of targeting to FAs (Figure 7). Similarly, the percentage of cells demonstrating FA localization of the LIM2^{T403V} mutant was not significantly different than those expressing wild-type avian paxillin at each of the time points analyzed (Figure 8A). However, the LIM2^{T403E} mutant showed an enhanced initial rate of localization to FAs relative to wild-type paxillin at 30 min (Figure 8A). This effect was not evident at the later time points, suggesting that phosphorylation of LIM2^{T403} facilitates the initial FA localization.

Several observations are noteworthy when the same strategy was employed to analyze the role of LIM3 serine phosphorylation in the rate of localization of paxillin to FAs (Figure 8B). Mutation of LIM3^{S457} to either nonphosphorylatable or phospho-mimetic residues had no effect on FA localization at 30 min. A generalized decrease in targeting rate was observed at later time points. This was not attributable to a perturbing effect on zinc-finger structure, since a molecule containing a zinc-chelating mutation of one finger of LIM3, LIM3^{C467A}, displayed wild-type localization by 15 h (our unpublished observations). This distribution is also reflected in the poor FA staining of these mutants observed at 24 h (Figure 6). In contrast, mutation of the second phosphorylated serine residue of LIM3 to alanine, LIM3^{S481A}, significantly retarded the rate of FA localization of paxillin at 30 min, whereas

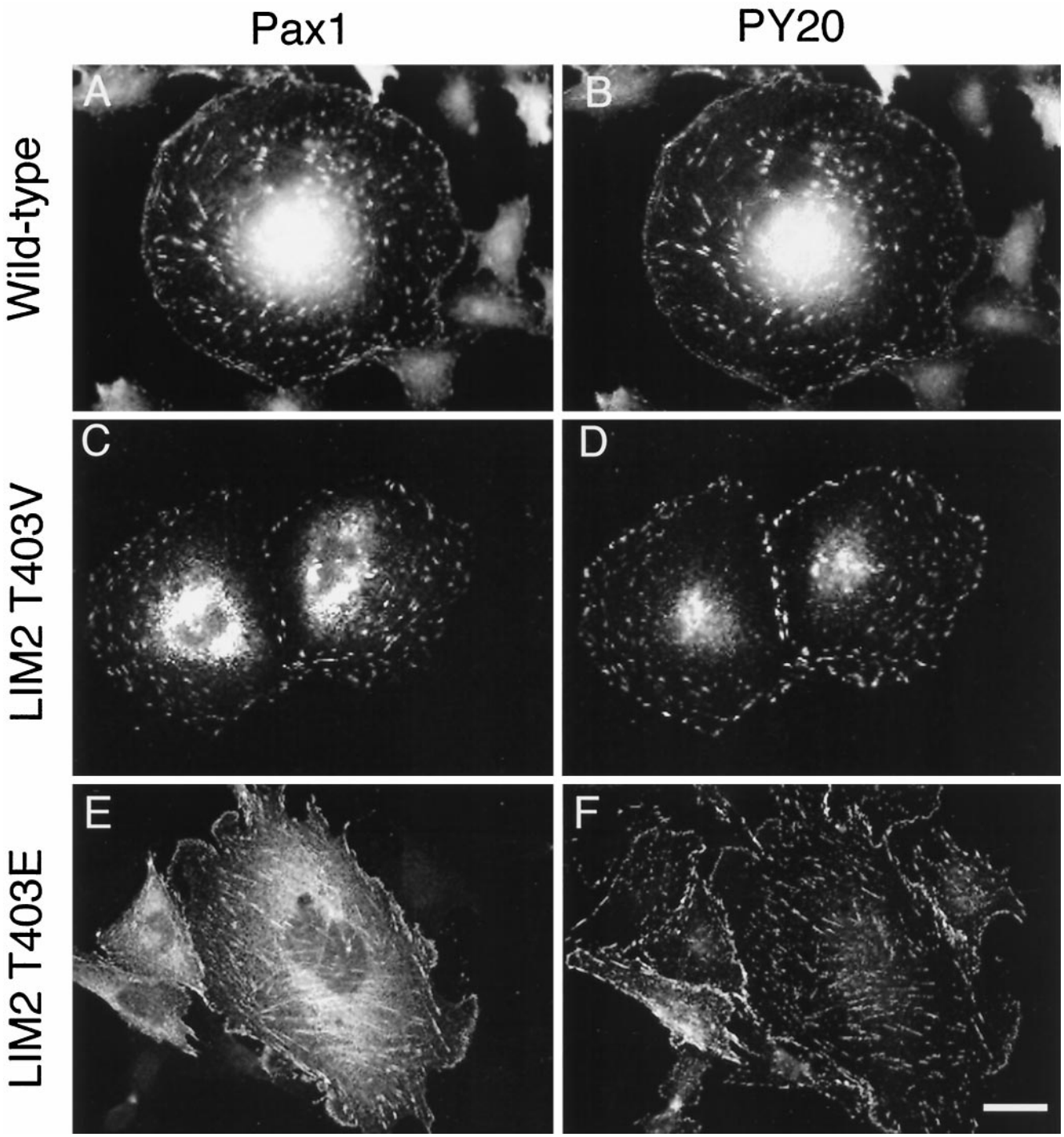

Figure 5. Immunofluorescence analysis of the capacity and efficiency of paxillin LIM2 phosphorylation mutants to localize to FAs. CHO.K1 cells were transfected with avian paxillin cDNA containing mutations of LIM2. After 24 h of growth on glass coverslips in Ham's F-12 media containing 10% FBS, ectopically expressed avian paxillin was visualized by immunofluorescence double-labeling with a chicken-specific, polyclonal antiserum Pax1 (A, C, and E) and a monoclonal antibody to phosphotyrosine, PY20 (B, D, and F). (A and B) Wild-type; (C and D) $LIM2^{T403V}$; (E and F) $LIM2^{T403E}$ are representative of the transfected populations. Bar, 5 μm.

the corresponding phospho-mimetic mutant, LIM3^{S481D}, showed markedly increased FA localization at 30 min (Figure 8C). These differences were statistically significant. This effect was restricted to the early time point, as the percentage of LIM3^{S481D} cells demonstrating FA localization equaled that of wild-type at 2 and 15 h. These data suggest phosphorylation of the LIM domains of paxillin regulates the subcellular distribution of paxillin in a temporal manner.

Role of LIM Domain Phosphorylation in Adhesion to Fibronectin

Determination of the mechanisms of regulating cellular adhesion is an area of intensive investigation. "Affinity modulation" of integrin binding to extracellular matrix is one means of precisely regulating integrin activity (O'Toole *et al*., 1990). Signals emanating from within the cell are involved in affinity modulation in a pathway termed "inside-out" signal transduction (reviewed by Ginsberg *et al.*, 1992). As paxillin phosphorylation changes are closely linked to changes in cytoskeletal organization and cell adhesion, paxillin is a candidate for participating in inside-out signaling. Consequently, we examined a role for the phosphorylated paxillin LIM domain residues in adhesion to a fibronectin substratum using CHO.K1 clones expressing avian paxillin molecules in fibronectin adhesion assays (Figures 9 and 10).

Adhesion of mutant avian paxillin-expressing clones was examined relative to wild-type avian paxillin-expressing cells. Analysis of Y31/118F and S188/190A mutants revealed no difference in adhesion with respect to wild-type, consistent with the FA localization data (Figure 9). In contrast, a significant alteration in cell adhesion was observed in cells expressing avian paxillin containing a mutation of LIM2^{T403}. Expression of the nonphosphorylatable LIM2^{T403V} protein caused a 25% reduction in the capacity of cells to adhere to fibronectin; whereas the LIM2^{T403E} phospho-mimetic stimulated adhesion by 25% (Figure 10A).

Similarly, blocking phosphorylation of LIM3^{S481} reduced the capacity of cells to adhere to fibronectin by 25%, while expression of the LIM3^{S481D} phospho-mimetic mutation stimulated adhesion to fibronectin by 25% (Figure 10C). Mutation of LIM3S457 to either a nonphosphorylatable or phospho-mimetic residue had no effect on cell adhesion (Figure 10B). These data directly correlate with the FA localization rates of the respective mutants and implicate paxillin as a component of inside-out regulation of cell adhesion through LIM domain phosphorylation.

DISCUSSION

Paxillin is a dynamically phosphorylated cytoskeletal molecular adaptor molecule that may participate in FA assembly and disassembly and modulate signaling from the FA. Altered states of phosphorylation within a cell affect cytoskeletal assembly and adhesion to the extracellular matrix (Burridge, 1986; Burridge *et al*., 1988; Burridge and Chrzanowska-Wodnicka, 1996). For instance, paxillin and FAK tyrosine phosphorylation states are modulated during integrin-mediated adhesion to the extracellular matrix and during cell migration (Burridge *et al*., 1992; Cary *et al*., 1996; Aznavoorian *et al*., 1996). A role for serine/threonine phosphorylation in adhesion and migration, although less well studied, is becoming apparent. Transient phosphorylation on serine 790 of the β_1-integrin cytoplasmic tail occurs during cell detachment. Mutation of this serine residue to the phospho-mimetic residue aspartic acid results in an elimination in the capacity of the integrin to localize to FAs (Barreuther and Grabel, 1996). Additionally, the serine/threonine kinase PKC localizes to FAs (Jaken *et al*., 1989) and is involved in FA formation and cell migration (Woods and Couchman, 1992; Derman *et al*., 1997). Further, several serine/threonine kinases that localize to FAs have been described recently including the β_1-integrin binding kinase ILK, p65 ^{PAK}, and p190 $^{ROK\alpha}$ (Hannigan *et al*., 1996; Harden *et al*., 1996; Leung *et al*., 1996). Moreover, rapid integrin-dependent serine phosphorylation of paxillin occurs during macrophage adhesion to vitronectin and fibroblast adhesion to fibronectin (De Nichilo and Yamada, 1996; Bellis *et al*., 1997). In these two examples, phosphoserine accounted for 95% of paxillin phosphorylation. We have identified serine residues 188 and 190 within the amino terminus as major targets of phosphorylation in response to adhesion to fibronectin (Bellis *et al*., 1997). However, in the current study, mutation of these serine residues, or tyrosine residues 31 and 118, had no effect on paxillin targeting to FAs or adhesion of CHO.K1 cells to fibronectin (Figures 7 and 9). Thus, the role of these residues remains to be determined.

The carboxyl-terminal LIM domains may function autonomously as a FA-targeting motif (Brown *et al*., 1996), positioning paxillin at the cell–matrix interface where the amino-terminal domains, potentially unconstrained, are free to effect signaling from the cell surface. Thus, regulation of paxillin LIM domain func-

Figure 6 (facing page). Mutation of paxillin LIM3 phosphorylation sites causes decreased efficiency of FA localization. CHO.K1 avian paxillin transfectants were grown on glass coverslips for 24 h in Ham's F-12 containing 10% FBS, followed by immunofluorescence double-labeling with a chicken-specific, polyclonal antiserum Pax1 (A, C, E, and G) and a monoclonal antibody to phosphotyrosine, PY20 (B, D, F, and H). (A and B) LIM3^{S457A}; (C and D) LIM3^{S481A}; (E and F) LIM3^{S457D}; (G and H) LIM3^{S481D} are representative of the transfected populations. The LIM3^{S457D} and LIM3^{S481A} mutant molecules showed a reduced colocalization of mutated paxillin and PY20 (see arrows). Bar, 5 μm.

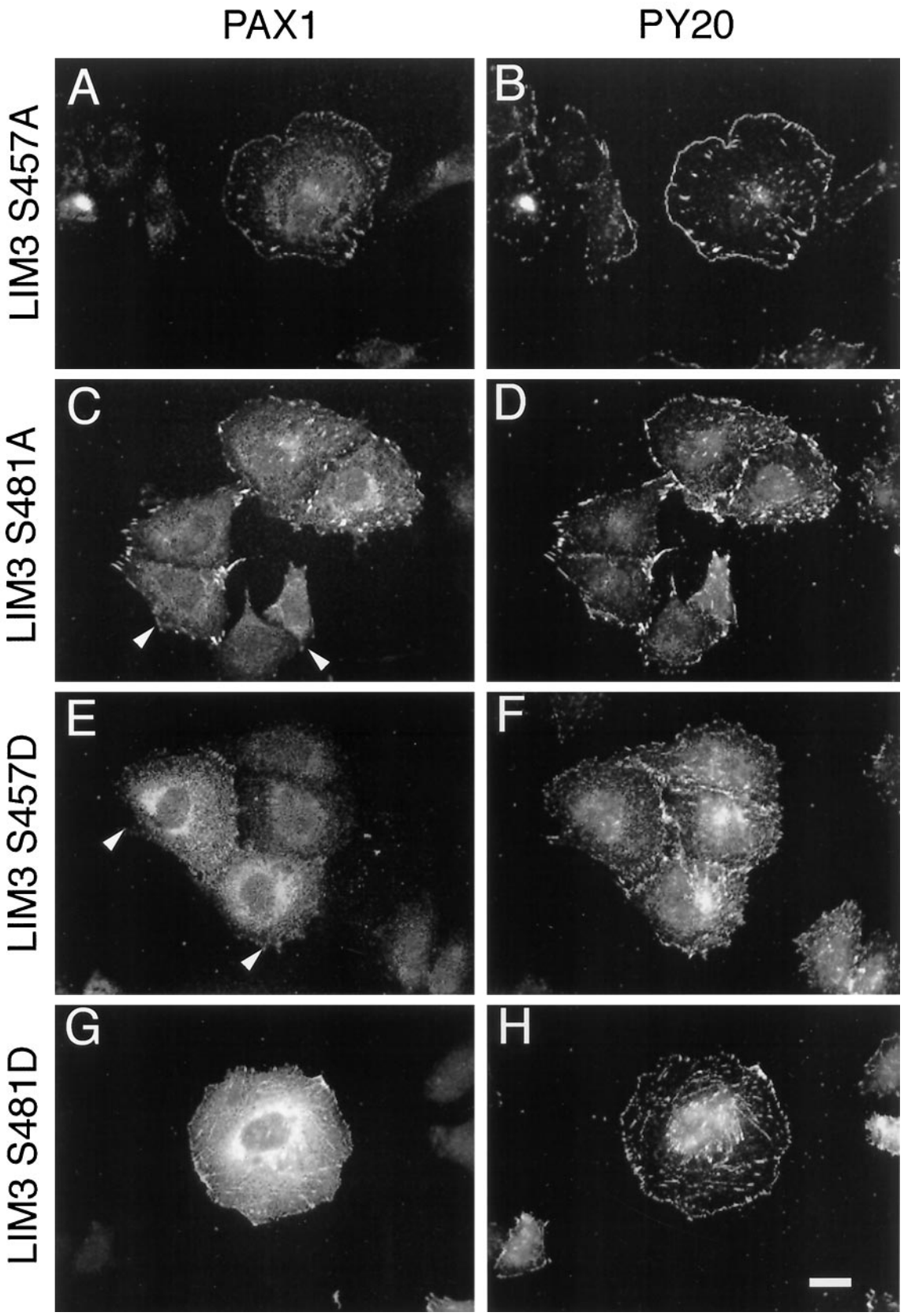
PAX1
PY20
LIM3 S457A
LIM3 S481A
LIM3 S457D
LIM3 S481D
A
B
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Figure 6.

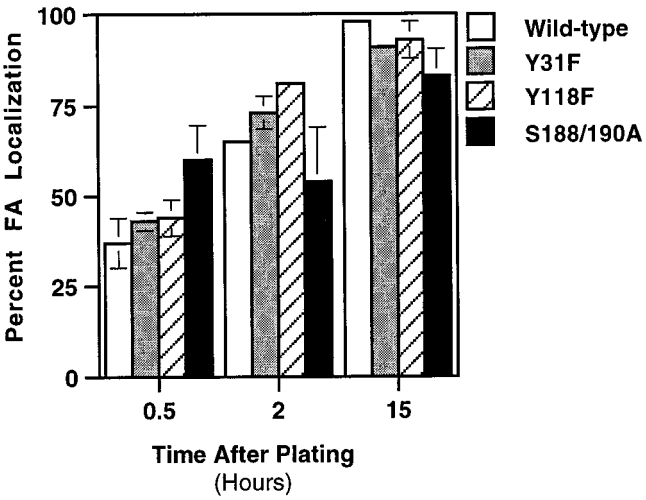

Figure 7. Localization rate of paxillin molecules containing mutations of the fibronectin-inducible phosphorylation sites Y31/118 and S188/190 is comparable to cells expressing wild-type avian paxillin. CHO.K1 fibroblasts expressing avian paxillin were maintained in suspension for 1 h before plating onto duplicate glass coverslips coated with 10 μg/ml fibronectin. At 0.5, 2, and 15 h, the coverslips were processed for immunofluorescence double-labeling using a chicken-specific polyclonal antiserum (Pax1) and either PY20 (Transduction Labs, Lexington, KY) to label FAs, or rhodamine-phalloidin (Molecular Probes, Eugene, OR) to decorate actin stress fibers. At each time point 150–200 transfected cells were counted with the number of avian paxillin transfectants displaying FA localization of the avian paxillin determined and divided by the total number of transfected cells. This is represented in bar graph form as the "Percentage localization to FA." All experiments were performed in duplicate with at least three independent experiments executed and tabulated to determine mean and SD from the mean. Statistical analyses were performed with Student's *t* test; *, $p < 0.05$; **, $p < 0.01$.

tion may serve as a major mechanism of modulating paxillin-dependent signals transduced through the highly integrated integrin and growth factor-signaling pathways (Plopper *et al.*, 1995; Rozengurt, 1995; Schwartz, 1997).

In the current study, we identify the binding of serine/threonine kinases to paxillin LIM2 and LIM3 (Figure 2). A family of LIM domain-containing serine/threonine protein kinases (LIMK) has been described although no known consensus sequence or physiologic substrates have yet been identified (Cheng and Robertson, 1995; Okano *et al.*, 1995; Pröschel *et al.*, 1995). We are currently investigating the possibility that members of the LIMK protein kinase family are directed to paxillin through a LIM–LIM interaction. Although specific isoforms of PKC associate with LIM domain structures (Kuroda *et al.*, 1996), a variety of PKC inhibitors had no effect on in vitro phosphorylation of either LIM2 or LIM3 (our unpublished observations).

The paxillin LIM-associated kinase activities are stimulated in CHO.K1 cells upon adhesion to fibronectin similar to the induced phosphorylation of tyrosines 31 and 118 and serines 188 and 190. However, unlike the amino-terminal residues, we determined that the LIM domain phosphorylation sites identified in vitro regulate paxillin localization to FAs after adhesion to fibronectin. Constitutive phosphorylation of paxillin ($LIM2^{T403E}$) significantly enhanced the FA-targeting rate after 30 min on fibronectin, as compared with wild type. This elevation was transient. A potential explanation is that phosphorylation of $LIM2^{T403}$ "primes" rapid paxillin localization to newly forming FAs and then participates in the process of FA formation. This may explain the detection of constitutive LIM2 kinase activity in CHO.K1 cells maintained in suspension. However, the fact that blocking phosphorylation of T403 (T403V) did not abrogate targeting indicates phosphorylation of $LIM2^{T403}$ is not essential for the directing of paxillin to FAs.

Analysis of LIM3 phosphorylation revealed that preventing phosphorylation of $LIM3^{S481}$ substantially decreased localization of paxillin relative to wild-type after 30 min on fibronectin whereas mimicking phosphorylation, $LIM3^{S481D}$, resulted in a much greater rate of localization versus wild-type at 30 min. This suggests that transient phosphorylation of $LIM3^{S481}$ also potentiates targeting of paxillin to newly forming FAs.

Clearly, phosphorylation of the LIM domains regulates paxillin FA localization, thus defining a novel mechanism of regulating LIM function. One possible mechanism is that LIM phosphorylation in response to cell stimulation alters the conformational profile of the four LIM domains and affects the presentation of the FA-targeting motif as well as other regions of the molecule that interact with as yet unidentified molecules. Such a phosphorylation-regulated paradigm is illustrated by the regulation of cyclin B1 activity. Cyclin B1 in a nonphosphorylated state is inactive and is maintained in the cytoplasm by a cytoplasmic retention signal (CRS) domain. Serine phosphorylation of residues within the cyclin B1 CRS results in a structural change that triggers nuclear localization. Mimicking phosphorylation by mutating the phosphorylated serine residues within the CRS to glutamic acid led to constitutive nuclear localization whereas blocking phosphorylation by mutation to alanine prevented the cytoplasmic to nuclear shuttle and cyclin B1 activity (Li *et al.*, 1997). An analogous phosphorylation-dependent alteration in localization is observed with the cytoskeletal protein talin. Changes in talin phosphorylation after phorbol ester or interleukin-1β stimulation regulates the capacity of talin to maintain a stable FA distribution (Turner *et al.*, 1989; Qwarnström *et al.*, 1991). Also, evidence of differential presentation of LIM-specific epitopes, depending on the cell activation state, has been found with the double-LIM do-

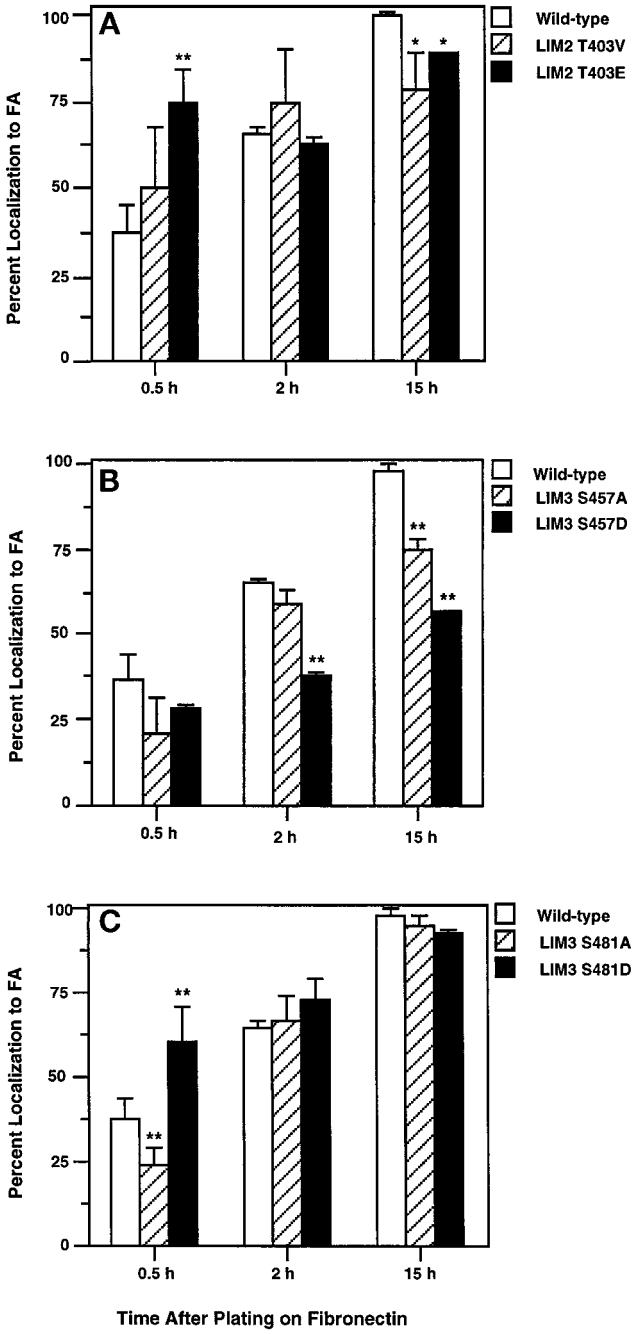

Figure 8. Increased FA localization rate of paxillin molecules containing phospho-mimetic mutations of phosphorylatable residues within LIM2 or LIM3. (A) $LIM2^{T403V}$ and $LIM2^{T403E}$. (B) $LIM3^{S457A}$ and $LIM3^{S457D}$. (C) $LIM3^{S481A}$ and $LIM3^{S481D}$. After adhesion to 10 μg/ml fibronectin-coated glass coverslips for 0.5, 2, or 15 h, the cells were processed and immunofluorescence double-labeled with Pax1 and PY20 or rhodamine-phalloidin. At each time point 150–200 transfected cells were counted with the number of avian paxillin transfectants displaying FA localization of the avian paxillin determined and divided by the total number of transfected cells. This is represented in bar graph form as the "Percentage localization to FA." Four independent experiments were executed and tabulated to determine mean and SD from the mean. Statistical analyses were performed with Student's *t* test; *, $p < 0.05$; **, $p < 0.01$.

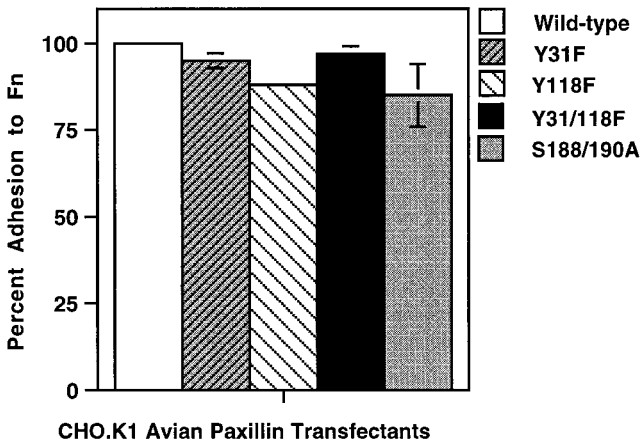

Figure 9. Adhesion of CHO.K1 cells expressing avian paxillin containing mutations of Y31/118F and S188/190A is identical to cells expressing wild-type avian paxillin. Avian paxillin transfectants were maintained in suspension for 1 h before adhesion for 30 min on 10 μg/ml fibronectin-coated, 1% BSA-blocked 96-well dishes, 8 wells per transfectant. After extensive washing, absorbance values were obtained by MTT assay, and adhesion relative to wild-type avian paxillin-expressing transfectants was calculated. Four independent experiments were executed, in duplicate, and tabulated to determine mean and SD from the mean. Statistical analyses were performed with Student's *t* test; *, $p < 0.05$; **, $p < 0.01$.

main proteins *Isl-1* and *LMO-1* (Lund *et al.*, 1995) although the mechanism is unknown.

Since LIM domain phosphorylation regulates paxillin FA localization, and paxillin is phosphorylated in response to cell adhesion, we examined a role for paxillin, and specifically, these phosphorylated residues, in inside-out signaling by measuring adhesion to fibronectin. Substantial alterations in CHO.K1 cell adhesion to fibronectin were induced by expression of avian paxillin proteins containing LIM domain phosphorylation mutations. By blocking phosphorylation of $LIM2^{T403V}$ or $LIM3^{S481A}$, cell adhesion was significantly reduced while constitutively phosphorylated $LIM2^{T403E}$ or $LIM3^{S481D}$ molecules potentiated cell adhesion to fibronectin.

The means by which paxillin regulates adhesion to fibronectin is not clear. Inside-out signal transduction encompasses a broad regulatory pathway that includes, but is not limited to, changes in integrin affinity, avidity and expression levels, as well as alterations in adhesive strength. Furthermore, the mechanisms and participants of inside-out signaling are ill-defined (O'Toole *et al.*, 1994; Stuiver and O'Toole, 1995). Both the α- and β-integrin subunit cytoplasmic domains are

Figure 8 (cont). determine mean and SD from the mean. Statistical analyses were performed with Student's *t* test; *, $p < 0.05$; **, $p < 0.01$.

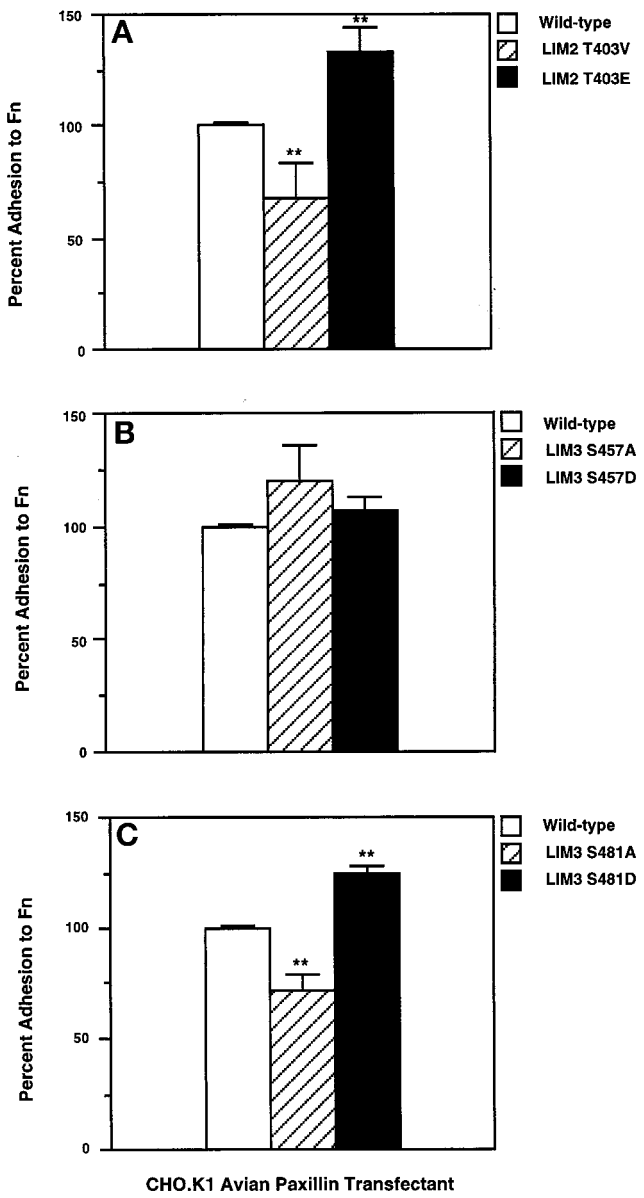

Figure 10. A role for the LIM domains of paxillin in regulating fibroblast adhesion to fibronectin. Blocking phosphorylation of T403 or S481 reduces cell adhesion by 25% whereas mimicking constitutive phosphorylation of these residues stimulates adhesion by 25%. (A) $LIM2^{T403V}$ and $LIM2^{T403E}$. (B) $LIM3^{S457A}$ and $LIM3^{S457D}$. (C) $LIM3^{S481A}$ and $LIM3^{S481D}$. Avian paxillin transfectants were maintained in suspension for 1 h before adhesion for 30 min on 10 μg/ml fibronectin-coated, 1% BSA-blocked 96-well dishes, 8 wells per transfectant. After extensive washing, absorbance values were obtained by MTT assay and adhesion relative to wild-type avian paxillin-expressing transfectants was calculated. Each graph represents the mean and SD from the mean from three adhesion assays, performed in duplicate. Statistical analyses were performed with Student's *t* test; *, $p < 0.05$; **, $p < 0.01$.

hypothesized to be associated with an "integrin activator complex" that can effect changes in integrin activity. Several FA proteins have been shown to directly bind β_1-integrin subunits, placing them in proximity to effect affinity modulation (Ginsberg *et al.*, 1995; Dedhar and Hannigan, 1996). Paxillin is also present in these complexes (Schaller and Parsons, 1995; Tanaka *et al.*, 1996). Future studies will address the level of action of paxillin LIM domain phosphorylation in the regulation of cell adhesion to fibronectin.

The capacity of paxillin to modulate cellular adhesion indicates that this FA molecule is not only a conduit of information from the extracellular environment to the intracellular signaling apparatus (a component of "outside-in" signaling), but also provides the first evidence that paxillin may contribute directly to the transmittance of signals from the cytoplasm to the external environment (inside-out signaling). In this regard, it will be important to determine whether paxillin LIM phosphorylation is involved in modulating cellular events dependent on cycling of integrin activity such as cell migration (Lauffenberger and Horwitz, 1996).

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