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Characterization of Paxillin LIM Domain-Associated Serine Threonine Kinases: Activation by Angiotensin II in Vascular Smooth Muscle Cells

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Abstract Recently we reported a novel means of regulating LIM domain protein function. Paxillin LIM zinc-finger phosphorylation in response to cell adhesion regulates the subcellular localization of this cytoskeletal adaptor protein to focal adhesions, and also modulates cell adhesion to fibronectin (Brown et al. [1998] Mol. Biol. Cell 9:1803–1816). In the present study, we characterize further the protein kinases that phosphorylate paxillin LIM2 on threonine and LIM3 on serine. Analysis of the subcellular distribution of the LIM kinases demonstrated that the LIM3 protein kinase, but not the LIM2 kinase, resides within a detergent-insoluble fraction. The activities of the paxillin LIM domain kinases are differentially regulated during embryogenesis, and analysis of tissue distribution indicated a specificity in expression patterns between the LIM2 and LIM3 kinases. In addition, these protein kinases were refractory to inhibition by a panel of broad-spectrum serine/threonine kinase inhibitors, suggesting a novel derivation. The paxillin protein kinase activities were stimulated in serum-starved CHO.K1 cells by the mitogen phorbol myristate acetate (PMA), and by PMA and angiotensin II in rat aortic smooth muscle cells. In vivo labeling, phosphoamino acid analysis, and phosphopeptide mapping of paxillin immunoprecipitated from angiotensin II-stimulated smooth muscle cells confirmed an induction of paxillin serine/threonine phosphorylation and supports the contention that these newly identified paxillin kinases are dynamic components of growth factor signaling through the cytoskeleton. J. Cell. Biochem. 76:99–108, 1999.

Key words: LIM domain; paxillin kinases; phosphoamino acid analysis; phosphopeptide mapping

Phosphorylation provides a rapid and specific means of controlling protein activity and is a principal regulatory mechanism for many proteins through an alteration in compartmentalization. Examples of phosphorylation-based regulation of subcellular distribution include the cytoskeletal elements talin, β-catenin, and paxillin [Turner et al., 1989; Miller and Moon, 1997; Brown et al., 1998]. Paxillin is a 68-kDa focal adhesion phosphoprotein that is localized to actin membrane attachment sites. The phosphorylation state of paxillin changes during cell adhesion, remodeling of the actin-based cytoskeleton, and during cell growth and differentiation [Zachary et al., 1993; Turner et al., 1994]. Coincident with paxillin tyrosine phosphorylation during these events is the activation of the tyrosine kinase FAK [Turner et al., 1993, 1995].

Although tyrosine phosphorylation events have been a principal focus of paxillin study, recent reports have detailed paxillin phosphorylation on serine/threonine in response to adhesion on vitronectin [DeNichilo and Yamada, 1996] and fibronectin [Bellis et al., 1997], interleukin-3 (IL-3) stimulation [Salgia et al., 1995] and after papillomavirus infection [Vande Pol et al., 1998]. Examination of the paxillin cDNA shows several potential serine/threonine phosphorylation sites as well as many other motifs implicated in protein-protein interactions [Turner and Miller, 1994; Salgia et al., 1995]. Multiple SH2- and SH3-binding domains, paxillin LD motifs, and LIM zinc-finger domains are contained within the primary amino acid sequence of paxillin [Turner and Miller, 1994; Brown et al., 1996]. LIM domains are cysteine/

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histidine-based double zinc fingers of approximately 50 amino acids that are present within a diversity of proteins, including members of the proto-oncogene *LMO* (LIM-only) family of helix-loop-helix transcriptional regulators, SH3-containing proteins, GTPase activating proteins, and a family of serine/threonine kinases [Gill, 1995; Jurata and Gill, 1998]. Paxillin is a member of a family of LIM-containing, cytoskeleton-associated focal adhesion proteins that includes the proteins zyxin, cysteine-rich protein (CRP) [Sadler et al., 1992], muscle LIM protein (MLP) [Arber and Caroni, 1996], and, more recently, enigma [Durick et al., 1998].

LIM domains function primarily in protein-protein interaction, rather than DNA-binding, through dimerization with other LIM domains [Schmeichel and Beckerle, 1994], binding to zinc fingers such as those of protein kinase C (PKC) [Kuroda et al., 1996], to tyrosine tight turn NPXY motifs [Wu and Gill, 1994; Wu et al., 1996], and non-tyrosine-based LIM interaction domains [Jurata and Gill, 1997]. The LIM domains of paxillin localize this protein to focal adhesions [Brown et al., 1996] and LIM domains direct CRP and MLP to the nucleus or along actin stress fibers [Arber and Caroni, 1996]. Taken together, these data suggest that LIM domains function in protein-protein interactions and target proteins to particular regions of the cell.

To identify and characterize modules on paxillin that may function in protein-protein interactions, we have used precipitation kinase assays to demonstrate the interaction of protein kinases with the LIM domains of paxillin. Previously we found that phosphorylation of the LIM domains of paxillin regulates the localization of paxillin to focal adhesions and potentiates cell adhesion to fibronectin [Brown et al., 1998]. In this study, the biochemical characteristics, as well as the developmental and tissue-specific expression of these paxillin LIM-associated serine/threonine kinases are investigated. We provide evidence that these potentially novel protein kinases are responsive to growth factor signaling and may be important components in the integration of growth factor and integrin signal transduction.

MATERIALS AND METHODS

GST-Paxillin Precipitation and In Vitro Kinase Assays

Glutathione-S-transferase (GST)-paxillin fusion proteins were generated and purified as previously described [Brown et al., 1998]. For kinase assays, tissue or cell lysates were prepared by homogenizing in 10 vol of lysis buffer containing 50 mM Tris-HCl pH 7.6, 50 mM NaCl, 1 mM EGTA, 2 mM $MgCl_2$, 0.1% β-mercaptoethanol, 1% Triton X-100, and a cocktail of protease inhibitors (Complete®, Boehringer Mannheim). The lysate was clarified at 14,500*g* for 15 min. Aliquots of lysate (1 mg of tissue or 250 µg cell lysate) were incubated with 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, washed extensively in lysis buffer, followed by boiling in 2× sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer for separation of proteins by SDS-PAGE, or 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 with 10 µCi of [γ-^{32}P]-ATP (ICN Pharmaceuticals, Irvine, CA). The phosphorylation reaction proceeded at room temperature for 20 minutes then was terminated by boiling directly in SDS-PAGE sample buffer. The reactions were processed by SDS-PAGE, stained with Coomassie blue to confirm equal fusion protein loading, dried and analyzed by autoradiography at −70°C, using Kodak X-OMAT film.

For thrombin cleavage of the LIM domains from GST, a precipitation kinase assay was performed, followed by adjustment of the kinase buffer to 2.5 mM $CaCl_2$ and the addition of 1 U of thrombin (Sigma Chemical Co., St. Louis MO). The site-specific cleavage reaction proceeded at 37°C for 5 min upon which the reaction was terminated by the addition of SDS-PAGE sample buffer and the sample was electrophoresed on a 17.5% acrylamide SDS-PAGE gel, followed by autoradiography.

For kinase assays involving pharmacologic inhibition, affinity isolation of protein kinases was performed as above, followed by the addition of protein kinase inhibitor (or vehicle) before the addition of the [γ-^{32}P]-ATP. The phosphorylation reactions and SDS-PAGE processing were performed as above. The concentrations of the inhibitors (Calbiochem) were as follows [IC_{50} in brackets]: 40 µM of the protein kinase C (PKC) inhibitor nonapeptide, polymyxin B ($K_i < 20$ µM); 100 µM of the PKC [6 µM], protein kinase A (PKA) [3 µM], protein kinase G (PKG) [5.8 µM], and myosin light chain kinase (MLCK) [97 µM] inhibitor H-7; 75 µM of

the PKC (31.7 μM), PKA (0.048 μM), PKG (0.48 μM), MLCK (28.3 μM), calmodulin-dependent protein kinase (CaMK) [29.7 μM], and casein kinase I (CKI) [38.3 μM] inhibitor H-89 [casein kinase II (CKII) K_i 137 μM]; or 15 μM of the PKA (4.3 μM), PKG (3.8 μM), MLCK (7.4 μM), and CKII (5.1 μM) inhibitor A3 [CKI K_i 80 μM, PKC 47 μM].

Cell Culture and In Vivo Labeling

CHO.K1 cells were cultured in modified Ham's F-12 (Mediatech, Washington, DC) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Gibco-BRL, Grand Island, NY, or Summit Biotechnologies, Ft. Collins, CO) and 1% penicillin-streptomycin at 37°C in a humidified chamber with 5% CO_2. For serum starvation, CHO.K1 were washed extensively in serum-free Ham's F-12, followed by 2 days of culture in serum-free medium, and then treated with 100 nM PMA (Sigma) for 10 min.

Rat aortic smooth muscle cells (RASM cells) in 100-mm tissue culture-treated dishes were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 25 mM Hepes, 10% (v/v) heat-inactivated FBS (Gibco-BRL, or Summit Biotechnologies), 2 mM L-glutamine, and 1% penicillin-streptomycin at 37°C in a humidified chamber with 5% CO_2. For serum starvation, cells were washed extensively in serum-free medium and cultured in defined serum-free medium (containing 25 mM Hepes, 5 μg/ml bovine holotransferrin, 20 nM selenium, 2 mM L-glutamine, and 1% penicillin-streptomycin) for 3 days and then treated with either 100 nM PMA or 1 μM angiotensin II (Sigma) for the indicated times.

For ^{32}P labeling, RASM cells were cultured and serum-starved as above then incubated for 4 h at 37°C in a humidified chamber with 5% CO_2 in phosphate-free DMEM (Gibco), supplemented with 25 mM Hepes, 2 mM L-glutamine, and 1% penicillin-streptomycin, followed by incubation for 4 h in the same medium supplemented with 1.5 mCi/ml ^{32}P-phosphoric acid (ICN Pharmaceuticals). Cells were then stimulated with 1 μM angiotensin II for 1 h. After extensive washing in ice-cold Earle's Balanced Salt Solution containing phosphatase inhibitors (1 mM sodium orthovanadate, 25 mM sodium fluoride, 25 mM β-glycerophosphate, 2 mM sodium pyrophosphate, 1 mM p-nitrophenylphosphate), the cells were lysed and boiled for 5 min in 500 μl denaturing immunoprecipitation lysis buffer with phosphatase inhibitors (1% SDS, 1% TX-100, 0.1% DOC, 20 mM Hepes 7.4, 150 mM NaCl, 2.5 mM EDTA, 10% glycerol). The lysates were pelleted at 14,500*g* for 15 min at 4°C, the supernatant was transferred to a fresh tube containing 1 ml standard immunoprecipitation buffer (1% Triton-X 100, 0.1% DOC, 20 mM Hepes 7.4, 100 mM NaCl, 1 mM EDTA), and precleared by a 30-min incubation with 50 μl 50% washed-Pansorbin® at 4°C end-over-end. The supernatant was incubated overnight at 4°C with 1 μl paxillin monoclonal antibody (clone #349, Transduction Laboratories), followed by precipitation with 25 μl protein A/G-agarose (Santa Cruz) for 2 h at 4°C end-over-end. The samples were processed by 10% SDS-PAGE, transferred to 0.45 μm Immobilon-P (Millipore) for trypsinization following standard 2-D phosphopeptide mapping procedures [Van der Geer and Hunter, 1994], using 0.1-mm cellulose-coated 20 × 20 cm TLC plates (#5716, Merck, Darmstadt, Germany) without fluorescent indicator. Thin-layer electrophoresis was performed using pH 1.9 buffer at 1 kV for 1 h at 16°C; and phosphobuffer for the thin-layer chromatography (TLC) second dimension for 10 h. Phosphoamino acid analysis was performed on material derived from SDS-PAGE gel slices (GST in vitro kinase assay) or PVDF (in vivo labeling) using pH 3.5 buffer, following standard procedures [Van der Geer and Hunter, 1994].

RESULTS

Phosphorylation of the LIM Domains of Paxillin

The C-terminus of the focal adhesion protein paxillin is composed of four LIM domains that contain multiple protein kinase phosphorylation consensus sites [Turner and Miller, 1994]. Recently, we determined that the LIM domains serve as binding sites and substrates for serine/threonine kinases [Brown et al., 1998]. In this report, we used the precipitation kinase assay to further characterize the LIM domain phosphorylation events. The individual LIM domains of paxillin, expressed as GST-fusion proteins and purified on glutathione-Sepharose 4B beads, were used in solid-phase binding assays by incubating with chicken gizzard smooth muscle lysate. After washing extensively to remove nonspecifically bound proteins, the GST-LIM fusion proteins, and specifically precipitated proteins, were subjected to in vitro kinase assay as described under Materials and Methods. Phosphorylation reactions were analyzed by SDS-PAGE on 10% acrylamide gels. Phos-

phorylation events were detected after autoradiography of the dried SDS-PAGE gels. Consistent with our previous report [Brown et al., 1998], the GST-LIM2 and GST-LIM3 fusion proteins were phosphorylated (Fig. 1, left), whereas GST, GST-LIM1, and GST-LIM4 were not.

To confirm the nature and specificity of the putative LIM2 and LIM3 phosphorylation events, a precipitation kinase assay was performed, followed by thrombin cleavage as detailed under Materials and Methods. Two bands were apparent on the resulting autoradiogram, the GST-LIM2 fusion (approximately 43 kDa), as well as the liberated LIM2 (5.5 kDa). A radiograph band at 32 kDa, which would represent a phosphorylated GST moiety, was absent (Fig. 1, middle). An identical result was observed using GST-LIM3 (Fig. 1, middle). Phosphoamino acid analysis demonstrated that GST-LIM2 was phosphorylated on threonine and GST-LIM3 was phosphorylated on serine (Fig. 1, right). Previously, threonine 403 of LIM2, and serines 457 and 481 of LIM3, were localized as the target sites [Brown et al., 1998]. Thus, paxillin LIM2 and LIM3 recruit and serve as substrates for serine/threonine protein kinases.

Widespread Embryologic and Tissue Distribution of the LIM-Associated Protein Kinases

Since paxillin phosphorylation has previously been shown to be developmentally regulated [Turner et al., 1993], we examined the potential developmental regulation of these paxillin LIM2 and LIM3 kinase activities. Whole avian embryos at 2-day intervals from day 3 to day 11 were harvested with lysates prepared for examination by in vitro precipitation kinase assay. Paxillin LIM2 and LIM3 kinase activities were detectable at day 3. A profound increase in both activities was observed between day 3 and day 5, which was maintained up through embryonic day 9. Lysates prepared from day 11 embryos had abundant paxillin LIM2 kinase activity whereas total embryo LIM3 kinase activity was substantially reduced as compared with day 9 (Fig. 2, top).

The pattern of expression of the LIM-associated kinases was examined in several tissues derived from day 18 avian embryos. LIM2- and LIM3-kinase activity was precipitated from smooth, skeletal, and cardiac muscle tissues, as well as brain, liver, and bursa/spleen (Fig. 2, middle). Interestingly, almost no detectable LIM2-kinase activity was recovered in lung, whereas a LIM3-kinase signal was observed. The LIM2-kinase activity was more abundant than LIM3-kinase activity in skeletal and heart muscle, as well as in the liver (lanes 2, 3, and 5), whereas LIM3-kinase activity was more abundant in brain and lung (lanes 4, 7).

The paxillin LIM2- and LIM3-associated kinase activities demonstrated distinct developmental and tissue-specific expression patterns

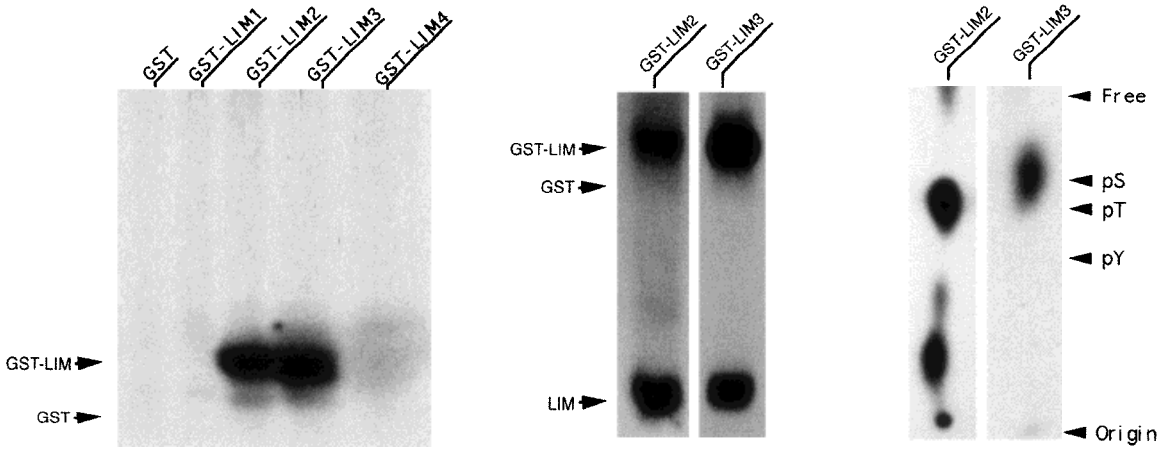

Fig. 1. Phosphorylation of the LIM2 and LIM3 domains of paxillin. **Left:** GST or GST-paxillin LIM domain fusion proteins conjugated to glutathione-Sepharose 4B beads were used to affinity isolate protein kinases from smooth muscle lysate, followed by in vitro kinase assay as described under Materials and Methods. **Middle:** GST-LIM2 or GST-LIM3 were phosphorylated as above, followed by thrombin cleavage to demonstrate phosphorylation of the fusion protein on the LIM domain, rather than the GST portion of the fusion protein. **Right:** phosphoamino acid analysis of the phosphorylated GST-LIM2 and GST-LIM3 fusion proteins shows that phosphorylation was restricted to threonine and serine, respectively.

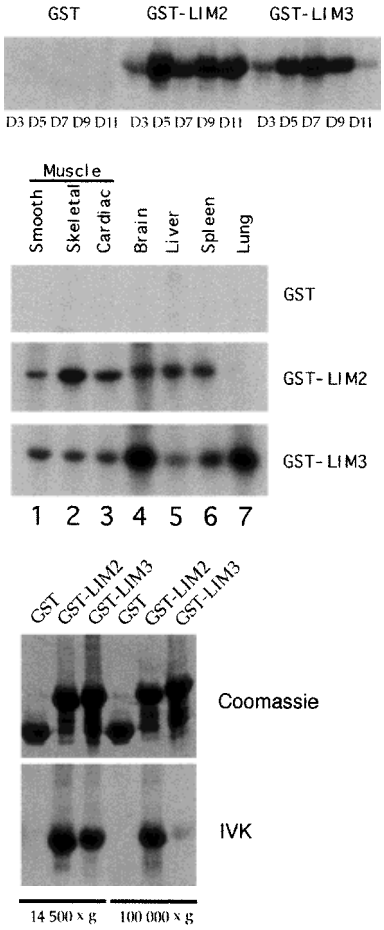

Fig. 2. Analysis of the developmental, tissue, and subcellular expression of the paxillin LIM-associated protein kinases. **Top:** developmental regulation of the LIM2- and LIM3-associated kinases was determined to increase during development with LIM2 kinase maintained, and LIM3 kinase activity curtailing, at day 11. **Middle:** the LIM kinases demonstrate widespread tissue distribution. Tissues were collected from day 18 chicken embryos, lysates were prepared, and in vitro kinase assays were performed using GST and GST-LIM fusion proteins. The sharp reduction in embryonic LIM3 kinase activity at day 11, and the low LIM2 kinase signal in lung tissue, suggest that the LIM2 and LIM3 kinases are distinct entities. **Bottom:** the LIM3-associated kinase is Triton X-100-insoluble. Smooth muscle lysates were prepared and subjected to 14,500*g* or 100,000*g* centrifugation steps, followed by examination of the supernatant by in vitro precipitation kinase assay (IVK).

(Fig. 2). To determine whether the serine/threonine kinase activities also exhibited differences in subcellular distribution we generated smooth muscle detergent extracts and subjected the lysates to sequential 14,500*g* and 100,000*g* centrifugation steps, followed by examination of the supernatants by in vitro precipitation kinase assay (Fig. 2, bottom). The paxillin LIM2-associated kinase was soluble, whereas the LIM3 kinase was precipitated by the 100,000*g* centrifugation step. This finding suggests that the serine kinase that binds to the paxillin focal adhesion localization motif, LIM3, is associated with the cytoskeleton.

Pharmacologic Characterization of the Paxillin LIM Domain Phosphorylation Events

The LIM2 domain of paxillin contains two threonine residues that fall into a weak PKC or cyclic nucleotide-dependent protein kinase consensus, whereas LIM3 has two serine residues that resemble a weak CK2 consensus [Songyang et al., 1994, 1996]. To determine whether these kinases were responsible for the in vitro phosphorylation events, GST-LIM2, or GST-LIM3 were incubated with chicken smooth muscle lysate, washed extensively, followed by precipitation kinase assay in the absence or presence of polymyxin B, H-7, H-89, or A3. Polymyxin B selectively inhibits cPKC. H-7 was used at a concentration that would inhibit cPKC, PKA, PKG, and MLCK; H-89 at a concentration that would target PKC, PKA, PKG, MLCK, CaMK, and CKI, and A3 was used at a concentration that would inhibit PKA, PKG, MLCK, and CKII (see under Materials and Methods). As shown in Figure 3, the addition of these inhibitors failed to eliminate LIM2 or LIM3 phosphorylation. However, incubation with polymyxin B slightly stimulated LIM2- and LIM3-precipitated kinase activity (Fig. 3, lane 2), whereas the addition of A3 or H-7 resulted in a modest reduction of paxillin LIM2 domain phosphorylation, and an approximately 40% reduction in LIM3 phosphorylation (Fig. 3, lanes 3, 4). Incubation with higher concentrations of the inhibitors did not result in a greater reduction of phosphorylation (our unpublished observations), suggesting that CaMK, CK, MLCK, cPKC, PKA, or PKG are unlikely to be the paxillin LIM2 and LIM3-associated kinases.

Phosphorylation of the LIM Domains of Paxillin In Vivo

To probe the potential significance of paxillin LIM domain phosphorylation events in a physiologic context, we used cultured mammalian cells to examine the sensitivity to mitogens of the LIM-kinase activities. CHO.K1 cells that had been serum starved were stimulated with phorbol 12-myristate 13-acetate (PMA) (Fig. 4). Very little LIM2- or LIM3-kinase activity was precipitated from adherent, serum-starved

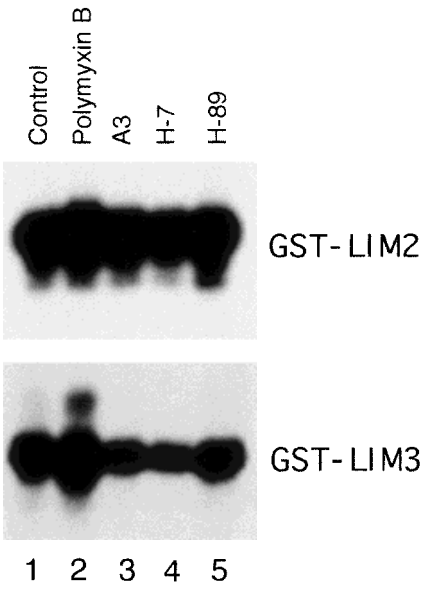

Fig. 3. Pharmacologic characterization of LIM2 and LIM3 phosphorylation in vitro. GST-LIM2 was incubated with smooth muscle lysate and washed, followed by kinase assay in the absence or presence of a panel of protein kinase inhibitors. GST-LIM3 was incubated with smooth muscle lysate, washed, followed by kinase assay in the absence or presence of a panel of protein kinase inhibitors. None of these inhibitors resulted in a >40% reduction of the GST-LIM2- or GST-LIM3-precipitated kinase activity.

CHO.K1 (lanes 2, 3), whereas activation of CHO.K1 with PMA induced both precipitated LIM-kinase activities (lanes 5, 6).

Next we examined RASM cell primary cultures for the presence of LIM kinase activity. It has been established that stimulation of serum-starved quiescent RASM cells with PMA or the vasoactive hormone angiotensin II stimulates tyrosine phosphorylation of the focal adhesion proteins FAK and paxillin, which coincides with a rapid reorganization of the cytoskeleton [Turner et al., 1995]. Precipitation kinase assays using lysates derived from confluent cultures of serum-starved RASM cells stimulated with either PMA or angiotensin II showed an induction of both the LIM2- and LIM3-kinase activities (Fig. 4). A time course of angiotensin II stimulation of LIM-kinase activity showed an increase in serine/threonine kinase activity that was sustained for at least 60 min (Fig. 4), a timepoint in which angiotensin II-stimulated paxillin tyrosine phosphorylation declines [Turner et al., 1995]. These data suggest that paxillin phosphorylation on threonine and serine plays a role in the dynamic alterations in

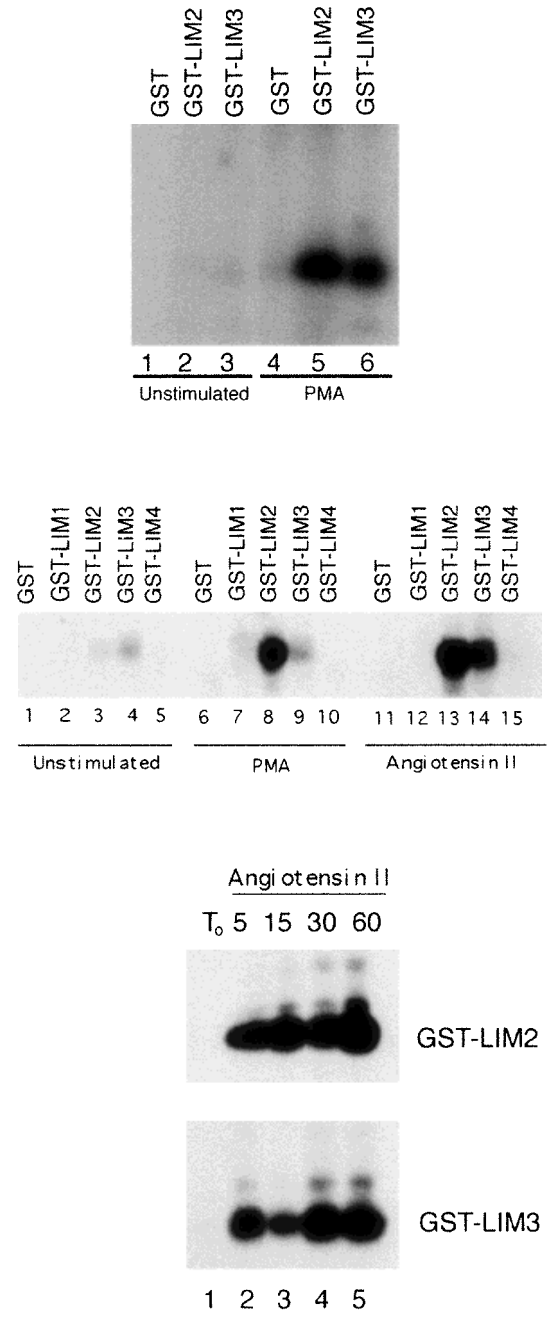

Fig. 4. Stimulation of the paxillin LIM2 and LIM3-associated protein kinases in fibroblasts and smooth muscle cells. **Top:** Serum-starved CHO.K1 cells were stimulated for 10 min with 100 nM PMA. Lysates were prepared and incubated with GST or GST-LIM fusion proteins, and an in vitro kinase assay was performed. **Middle:** Serum-starved RASM were stimulated for 10 min with 100 nM phorbol myristate acetate (PMA) or 1 μM angiotensin II. Lysates were prepared and incubated with GST or GST-LIM fusion proteins, and an in vitro kinase assay was performed. **Bottom:** A time course of stimulation of the LIM2- and LIM3-associated kinases in RASM by angiotensin II demonstrated a rapid and sustained activation of the LIM2 and LIM3 kinases.

smooth muscle cell functions associated with the renin-angiotensin system.

To confirm that angiotensin II was capable of inducing paxillin serine/threonine phosphorylation in RASM cells, we stimulated ^{32}P-labeled cells, followed by paxillin immunoprecipitation. As shown in Figure 5, one hour of angiotensin II treatment stimulated a significant increase in ^{32}P incorporation into paxillin. Phosphoamino acid analysis of the labelled paxillin revealed a significant increase in serine and threonine phosphorylation, with a marginal increase in phosphotyrosine content relative to unstimulated (Fig. 5). This demonstrated that angiotensin II stimulated paxillin serine/threonine phosphorylation in vivo. To determine whether there was an increase in the stoichiometry of paxillin serine/threonine phosphorylation, or whether novel sites of phosphorylation were targeted, a two-dimensional tryptic map was generated with paxillin derived from unstimulated and angiotensin II-stimulated RASM cells. Indeed, two novel sites of phosphorylation were induced on paxillin, suggesting that angiotensin II may trigger an activation of the paxillin LIM-associated serine/threonine kinases in vivo (Fig. 5, asterisks).

DISCUSSION

Paxillin is a cytoskeletal molecular adaptor molecule that may participate in the dynamic assembly and disassembly of focal adhesions and modulate the organization of and signalling from focal adhesions. We have been cataloging the protein-protein interaction domains of paxillin in order to gain insight into paxillin function and have identified and characterized the capacity of LIM2 and LIM3 to recruit and serve as substrates for two distinct serine/threonine kinases. The serine/threonine residues we have identified are intact across species and paxillin superfamily members, including paxillin-αβγ, leupaxin, and Hic-5. The kinase activities were found to have a widespread, but not ubiquitous, distribution, suggesting an important role for these phosphorylation events in regulating paxillin function, potentially in a cell type-specific manner. This is consistent with the apparent cell type- and tissue-specific expression of paxillin family members [Hagmann et al., 1998] and with prior studies demonstrating the developmental regulation of paxillin phosphorylation [Turner et al., 1993].

Although LIM domain phosphorylation is not a prerequisite for paxillin focal adhesion targeting, we found that paxillin LIM domain phosphorylation modulates the efficiency of paxillin localization to focal adhesions [Brown et al., 1998]. Furthermore, LIM domain phosphorylation potentiates the capacity of cells to adhere to fibronectin, whereas blocking phosphorylation significantly impairs fibronectin adhesion [Brown et al., 1998]. The identification of the

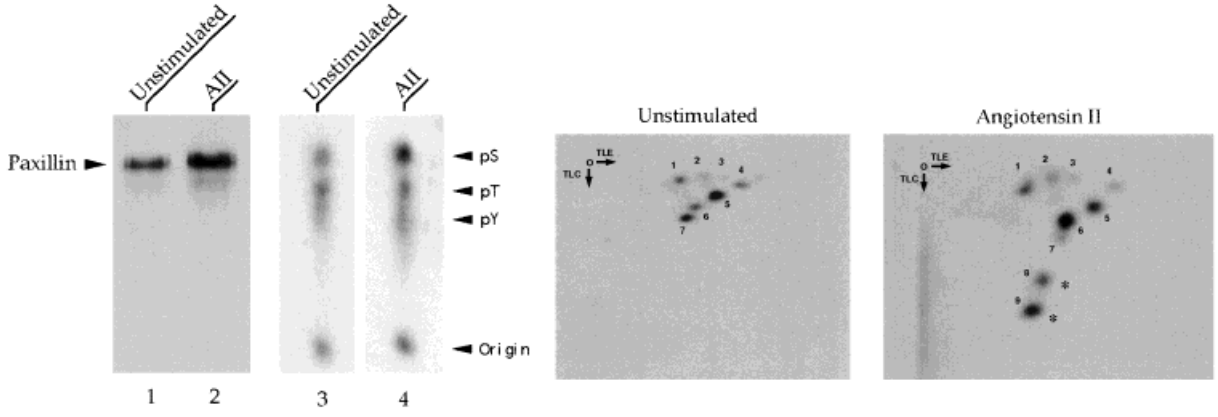

Fig. 5. In vivo stimulation of paxillin serine/threonine phosphorylation by angiotensin II. RASM cells were serum-starved for 2 days, labeled with ^{32}P-orthophosphate for 4 h, and then stimulated with 1 μm angiotensin II for 1 h, followed by paxillin immunoprecipitation and analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography (**lanes 1, 2**). Angiotensin II stimulated an increase in paxillin phosphorylation with one-dimensional phosphoamino acid analysis of in vivo ^{32}P-labeled paxillin, showing that angiotensin II stimulated an increase in serine/threonine phosphorylation (**lanes 3, 4**). Two-dimension tryptic peptide mapping of in vivo-labeled paxillin demonstrated that angiotensin II-stimulated the phosphorylation of novel sites of phosphorylation (spots 8 and 9, asterisks) as well as a "remodeling" of basal sites of phosphorylation (spots 1–7).

serine/threonine kinases, as well as the LIM3 binding protein involved in focal adhesion localization of paxillin, will provide greater insight into the precise mechanisms by which phosphorylation regulates paxillin function and signal transduction mediated through this protein. Our identification of phosphorylation of the paxillin LIM domains may be evidence of a more widespread mechanism of LIM protein regulation, as phosphorylation of the LIM-containing proteins zyxin, abLIM, and leupaxin has also been reported.

The inability of a panel of serine/threonine kinase inhibitors to eliminate the LIM domain phosphorylation may indicate that the kinases precipitated from smooth muscle are novel in nature (Fig. 3). A large family of LIM-domain-containing serine/threonine protein kinases has been described [Okano et al., 1995]. LIM-kinase (LIMK or KIZ) is an actin-binding protein that phosphorylates cofilin to mediate cytoskeletal reorganization [Arber et al., 1998; Yang et al., 1998]. As LIM domains can functionally dimerize [Gill, 1998], it is interesting to speculate that members of this protein kinase family may be directed to paxillin through interaction with the paxillin LIM domains. However, it has been reported that LIMK is inhibited by PMA treatment [Arber et al., 1998], whereas the paxillin LIM-associated kinase activities are stimulated by PMA (Fig. 4). Other potential paxillin binding and targeting partners include cytoskeletal LIM-family proteins and tyrosine-containing tight turn NPXY motifs that are present on the cytoplasmic tails of many transmembrane receptors such as the β-integrins [Reszka et al., 1992], the insulin and epidermal growth factor receptor (EGFR) tyrosine kinases [Trowbridge et al., 1993], and the type 1 angiotensin II receptor [Hunyady et al., 1995]. In addition, several cytoskeletal serine/threonine kinases have recently been characterized that may mediate phosphorylation. These include the integrin-linked kinase, ILK, as well as several p21-regulated kinases that have been implicated in the reorganization of the actin cytoskeleton and the formation of focal adhesions. Paxillin has been reported to bind to β-integrin cytoplasmic tail peptides in vitro [Schaller et al., 1995; Tanaka et al., 1996], preliminary reports suggest that paxillin binds directly to the EGFR cytoplasmic tail through a LIM-NPXY interaction (our unpublished observations), and we recently have determined that paxillin associates with PAK complexes [Turner et al., 1999]. Further work will define the precise targets of paxillin association and functional consequences.

Phosphorylation states have long been known to regulate cytoskeletal protein function, most notably the activities of the tyrosine kinases Src and FAK. A conformational change associated with phosphorylation of the focal adhesion protein vinculin has also been shown to regulate vinculin actin-binding potential [Weekes et al., 1996; Schwienbacher et al., 1996], and phosphorylation of talin, β-catenin, and paxillin have been associated with alteration in subcellular localization [Miller and Moon, 1997; Turner et al., 1989; Brown et al., 1998]. Interestingly, cell activation results in the differential presentation of LIM epitopes of the proteins rhombotin and Isl-1 [Lund et al., 1995]. Thus, paxillin LIM phosphorylation may act as a switch that regulates the conformational interplay between the LIM domains, the activity of the focal adhesion targeting motif, and the multiple protein-protein interaction domains present within this molecule. Definition of paxillin phosphorylation sites and protein recognition domains will allow for a careful examination of the potential for phosphorylation-regulated protein-protein interaction remodeling and consequent effects on cell adhesion-associated events. Particular emphasis will be placed on the potential role of paxillin in those overt changes that occur during the cardiovascular and renal pathophysiologic tissue reorganization associated with dysregulation of the renin-angiotensin system.

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