Adhesion of fibroblasts to fibronectin stimulates both serine and tyrosine phosphorylation of paxillin

Susan L. BELLIS*, Joseph A. PERROTTA, Michael S. CURTIS and Christopher E. TURNER†

Department of Anatomy and Cell Biology, State University of New York Health Science Center, 750 E. Adams Street, Syracuse, NY 13210, U.S.A.

Tyrosine phosphorylation of paxillin by the focal adhesion kinase (FAK) has been implicated as a signal transduction mechanism associated with cell adhesion and cytoskeletal reorganization. The potential role of serine phosphorylation of paxillin in these events has not been well characterized. In this study we have examined the phosphorylation profile of paxillin both *in vitro* and *in vivo*. By using glutathione S-transferase–paxillin fusion proteins in precipitation-kinase assays *in vitro* we observed that a fusion protein spanning amino acid residues 54–313 of paxillin, and containing a FAK-binding site, precipitated substantial serine kinase activity as well as FAK activity from a smooth-muscle lysate. Together these kinases phosphorylated paxillin on tyrosine residue 118, a site that has been identified previously as a target for FAK phosphorylation, and on serine residues 188 and/or 190. The binding site for the serine

kinase, the identity of which is currently unknown, was further mapped to residues 168–191 of paxillin. To assess the physiological relevance of these sites phosphorylated *in vitro*, the profile of paxillin phosphorylation *in vivo* stimulated by seeding fibroblasts on fibronectin was characterized. As expected, plating cells on fibronectin enhanced the tyrosine phosphorylation of paxillin. However, 96% of the phosphorylation of paxillin occurred on serine residues. Comparison by two-dimensional phosphopeptide analyses indicated that the major sites of tyrosine and serine phosphorylation detected in the assays *in vitro* co-migrate with phosphopeptides derived from paxillin phosphorylated *in vivo* in response to plating cells on fibronectin. These findings support a role for both tyrosine and serine kinases in the signal transduction pathway linking integrin activation to paxillin phosphorylation.

INTRODUCTION

Integrins are heterodimeric transmembrane receptors that bind to components of the extracellular matrix such as fibronectin (FN), vitronectin and laminin. The association of integrins with extracellular matrix ligands catalyses the recruitment of cytoskeletal proteins into structures called focal adhesions and simultaneously induces the reorganization and polymerization of actin stress fibres [1,2]. The formation of focal adhesions and stress fibres serves to stabilize and strengthen the adhesion of cells to the extracellular matrix.

In addition to providing a structural link between cells and the extracellular matrix, focal adhesions represent important sites for signal transduction (reviewed in [3–5]). Notably, several known signalling enzymes, including protein kinase C (PKC) [6,7], c-Src [8–10] and the non-receptor tyrosine kinase, focal adhesion kinase (FAK) [11–16], are localized to these sites. Integrin activation induces a substantial increase in the tyrosine phosphorylation of FAK, an event that is correlated with enhanced FAK activity [13,14]. Thus FAK is a likely candidate for mediating integrin-regulated signalling pathways. The focal adhesion protein, paxillin, is also phosphorylated on tyrosine in response to integrin ligation [15,17,18], and several studies suggest that paxillin is a substrate for FAK [19–21].

Although the consequences of FAK and paxillin phosphorylation have not been well defined, the tyrosine phosphorylation of focal adhesion proteins might be required for integrin-mediated cytoskeletal reorganization. One of the primary functions of phosphotyrosine residues is to provide docking sites for proteins that contain Src homology 2 (SH2) domains. It follows that the tyrosine phosphorylation of proteins such as paxillin might facilitate the formation of protein complexes that could be

essential for both the assembly of focal adhesions structures as well as the efficient processing of extracellular stimuli. In support of this hypothesis, treatment of fibroblasts with tyrosine kinase inhibitors blocks integrin-stimulated focal adhesion and stress fibre formation [15,22]. However, in contrast with this finding, it has been recently reported that FAK activity might play a more important role in cell migration than in focal adhesion assembly [23].

Concomitantly with the activation of tyrosine kinases such as FAK, multiple serine/threonine kinases are stimulated by the association of integrins with extracellular matrix ligands. In particular, both PKC and mitogen-activated protein kinase (MAPK) are activated in response to cell adhesion. Potential changes in the phosphoserine and phosphothreonine content of focal adhesion proteins in response to cell adhesion have not been well examined; therefore the specific substrates of integrin-activated serine/threonine kinases have not been delineated.

In the present study we have characterized the profile of paxillin phosphorylation that results from seeding fibroblasts on the integrin ligand, FN. We find that growing cells on FN stimulates tyrosine phosphorylation of paxillin as expected; however, a substantial increase in the phosphoserine content of paxillin was also observed. Experiments *in vitro* suggest that the serine kinase responsible for integrin-mediated paxillin phosphorylation binds with high affinity to paxillin.

EXPERIMENTAL

Synthesis of glutathione S-transferase (GST)–paxillin fusion proteins

Selected regions of paxillin cDNA [24] were amplified by PCR with oligonucleotides containing 5′ *Bam*HI or 3′ *Eco*RI re-

Abbreviations used: CEF, chicken embryonic fibroblast; FAK, focal adhesion kinase; FN, fibronectin; GST, glutathione S-transferase; MAPK, mitogen-activated protein kinase; PKC, protein kinase C; PL, polylysine.

* Present address: Department of Pathology, State University of New York Health Science Center, 750 E. Adams Street, Syracuse, NY 13210, U.S.A.

† To whom correspondence should be addressed.

striction sites. The PCR products were subcloned into *Bam*HI/*Eco*RI-digested pGEX-2T (Pharmacia). Verification that paxillin was subcloned in the correct reading frame was provided by dideoxy chain-termination sequencing (Sequenase 2, United States Biochemical). The GST–paxillin fusion proteins were expressed and purified by using GSH–Sephadex as described elsewhere [20,24].

Kinase assays *in vitro*

Gizzards from day 20 chicken embryos (a source of smooth-muscle focal adhesion proteins) were homogenized in 10 volumes of lysis buffer comprising 50 mM Tris/HCl, pH 7.6, 50 mM NaCl, 10 mM EGTA, 2 mM MgCl₂, 0.1 % Triton X-100 and 10 µg/ml leupeptin. Lysates were centrifuged at 100 000 *g* to remove insoluble material and were then incubated with various GST–paxillin fusion proteins coupled to GSH–Sephadex beads for 90 min at 4 °C with rotation. After the incubation, fusion proteins and associated lysate proteins were collected by centrifugation and washed extensively with lysis buffer to remove non-specifically bound proteins. Precipitates were resuspended into kinase buffer containing 10 mM Hepes, 3 mM MnCl₂ and 10 µCi of [γ -³²P]ATP. Kinase assays proceeded for 20 min at 25 °C and were terminated by the addition of SDS/PAGE sample buffer. Proteins were resolved by SDS/PAGE [25] and phosphorylated material was detected by autoradiography. Protein amounts were quantified with a Bio-Rad protein assay kit, as well as by Coomassie Blue staining of SDS/PAGE gels.

Kinase assays were also performed *in vitro* with lysates from chicken embryonic fibroblasts (CEFs) plated on either polylysine (PL) or FN. Briefly, CEFs grown to approx. 80 % confluence in serum-containing medium were detached from tissue culture dishes by treatment with trypsin. The cells were washed in serum-free Dulbecco's modified Eagle's medium (Gibco-BRL) then replated on tissue culture dishes precoated with either PL or FN (each at 25 µg/ml). After incubation for 4 h at 37 °C the cells were homogenized in lysis buffer (defined above) then centrifuged to remove insoluble material. Kinase assays were performed with CEF lysates and GST–paxillin fusion proteins as described above.

Site-directed mutagenesis

Point mutations in the paxillin sequence were generated by either a PCR-based method, as described by Landt et al. [26], or by standard procedures with the bacteriophage, M13 [27]. Mutated paxillin DNA was subcloned into pGEX-2T as described above. The sequences of all mutated fusion proteins were verified by dideoxy chain-termination sequencing.

Phosphopeptide mapping and phosphoamino acid analysis

Proteins phosphorylated in kinase assays *in vitro* were eluted from dried SDS/PAGE gels, treated with trypsin and subjected to two-dimensional phosphopeptide mapping by standard methods [28]. Phosphopeptides were separated in the first dimension by thin-layer electrophoresis in pH 1.9 buffer, then resolved by TLC in isobutyric acid buffer.

For phosphoamino acid analyses, phosphorylated proteins were excised from SDS/PAGE gels and hydrolysed in 6 M HCl at 110 °C for 90 min to release individual amino acids. Phosphoamino acids, with standards, were separated by thin-layer electrophoresis in pH 3.5 buffer [28].

Labelling *in vivo*

CEFs were detached from tissue culture dishes by treatment with trypsin, washed in serum-free, phosphate-free Dulbecco's

modified Eagle's medium and then seeded on tissue culture dishes precoated with either PL or FN. On replating, 0.8 mCi/ml of H₃³²PO₄ was added to the serum-free, phosphate-free media and cells were incubated for 4 h at 37 °C. After the radiolabelling procedure the cells were washed three times with PBS then lysed in 50 mM Tris/HCl (pH 7.5)/50 mM NaCl/10 mM EGTA/2 mM MgCl₂/0.1 % Triton X-100/10 µg/ml leupeptin/2 mM NaF/50 mM PP_i/2 mM sodium orthovanadate. Lysates were normalized by Cerenkov counting. Paxillin was immunoprecipitated from lysed cells with monoclonal antibody 165 [29] then resolved by SDS/PAGE. Elution of paxillin from gels and tryptic phosphopeptide analysis were as described above. The phosphorylation of paxillin *in vivo* was detected with a Fujix BAS2000 phosphorimager.

RESULTS

GST–paxillin precipitates both FAK and a serine kinase from a smooth-muscle homogenate

Our previous study demonstrated that a GST–paxillin fusion protein expressing amino acid residues 54–313 of paxillin (GST–paxillin 54–313) precipitated FAK from smooth-muscle lysates [24]. In view of this result we sought to identify potential sites of paxillin phosphorylation by FAK, as well as to determine whether other kinases could be precipitated by GST–paxillin 54–313. We therefore incubated GST–paxillin 54–313 with smooth-muscle lysates to allow the association of kinases with the fusion protein and then performed kinase assays *in vitro* with the washed GST–paxillin precipitates (see the Experimental section). Results from these kinase assays *in vitro* revealed the presence of three phosphorylated proteins with molecular masses of 55, 100 and 125 kDa (Figure 1A, lane 2). The 55 kDa protein represents GST–paxillin 54–313; the 125 kDa protein was identified as FAK by immunoblotting experiments (results not shown). The 100 kDa protein (p100) is of unknown identity and is currently being characterized. Kinase assays in which GST alone (Figure 1A, lane 1) was used to precipitate proteins from lysate did not yield phosphorylation of GST. Additionally, adding purified GST–paxillin 54–313 to GST that had been preincubated with lysate failed to result in phosphorylation of GST–paxillin 54–313 (results not shown). These results suggest that the phosphorylation of GST–paxillin 54–313 shown in Figure 1 results from the action of kinases that associate specifically with the paxillin domain, not the GST domain, of the GST–paxillin fusion protein.

Each of the three phosphorylated proteins was excised from an SDS/polyacrylamide gel and subjected to a phosphoamino acid analysis (Figure 1B). GST–paxillin 54–313 and the p100 protein were substantially phosphorylated on both serine and tyrosine residues, indicating that, in addition to FAK, a serine kinase was precipitated by GST–paxillin 54–313. In contrast, the radiolabelled band corresponding to FAK demonstrated phosphorylation on tyrosine residues only, suggesting that FAK is not a substrate for the precipitated serine kinase. The phosphorylation of FAK on tyrosine residues is most probably due to autophosphorylation [13,14].

To characterize further the specific sites of paxillin phosphorylation, two-dimensional phosphopeptide mapping experiments were performed with fusion proteins labelled *in vitro*. Two intensely labelled phosphopeptides were observed in the two-dimensional maps (Figure 2A). Phosphoamino acid analyses (results not shown) of these peptides indicated that peptide 1 contained phosphotyrosine and peptide 2 contained phosphoserine.

Figure 1 GST-paxillin 54–313 is radiolabelled on serine and tyrosine residues

(A) GST (lane 1) and GST-paxillin 54–313 (lane 2) were incubated with smooth-muscle lysates to allow the association of kinases. After the incubation these proteins were precipitated with GSH-Sepharose beads, the precipitates were washed and [γ - 32 P]ATP was added to initiate kinase assays *in vitro*. Phosphorylated proteins were resolved by SDS/PAGE and detected by autoradiography. The positions of molecular mass markers (in kDa) are indicated at the left. (B) The three radiolabelled proteins observed in (A), representing GST-paxillin 54–313, a 100 kDa protein (p100) and FAK respectively, were excised from SDS/PAGE gels and subjected to one-dimensional phosphoamino acid analyses by established procedures [28]. As shown, GST-paxillin 54–313 and the p100 protein were phosphorylated on both serine and tyrosine residues, whereas FAK was phosphorylated on tyrosine residues only. Abbreviations: PS, phosphoserine; PT, phosphothreonine; PY, phosphotyrosine.

The principal site of tyrosine phosphorylation of paxillin *in vitro* is Tyr-118

Previous studies of paxillin phosphorylation by FAK immunoprecipitates have demonstrated that a primary site of paxillin phosphorylation by FAK *in vitro* is Tyr-118 [20,21]. Because FAK was precipitated by GST-paxillin in the current investigation, it was likely that the principal phosphotyrosine-containing peptide represented phosphorylated Tyr-118. We therefore performed phosphopeptide mapping experiments with a fusion protein containing a phenylalanine substitution for Tyr-118. The two-dimensional phosphopeptide analysis of this fusion protein confirmed that radiolabelling of the major phosphotyrosine-containing peptide was due solely to the phosphorylation of Tyr-118 (Figure 2B).

Figure 2 Phosphopeptide analysis of GST-paxillin *in vitro*

(A) Radiolabelled GST-paxillin contains two major sites of phosphorylation. GST-paxillin 54–191 was phosphorylated *in vitro*, excised from SDS/PAGE gels and subjected to two-dimensional tryptic phosphopeptide mapping by established procedures [28]. Phosphoamino acid analysis of the two most intensely labelled tryptic peptides revealed one major site of tyrosine phosphorylation, peptide 1, and one major site of serine phosphorylation, peptide 2. GST-paxillin 54–191 has the same phosphopeptide profile as GST-paxillin 54–313 (see Figure 4A). (B) The major site of tyrosine phosphorylation is Tyr-118. GST-paxillin 54–191 containing a phenylalanine substitution for Tyr-118 (Y118F) was phosphorylated *in vitro* then subjected to phosphopeptide mapping. Compared with the two-dimensional map of wild-type GST-paxillin (A), the major phosphotyrosine-containing peptide was eliminated by introducing a phenylalanine substitution for Tyr-118 (B).

The principal site(s) of serine phosphorylation as well as the serine kinase-binding site reside within residues 168–191 of paxillin

GST-paxillin 54–313 contains 52 serine residues; it was therefore not practicable to use site-directed mutagenesis to identify specific sites of phosphorylation without first delineating the general region of serine phosphorylation. To begin mapping the region of paxillin containing the principal site(s) of serine phosphorylation, we synthesized GST-paxillin fusion proteins containing N-terminal and C-terminal truncations and used these in kinase assays *in vitro*. The phosphorylated fusion proteins were subjected to two-dimensional phosphopeptide mapping experiments to monitor the presence of the major phosphoserine-containing peptide. GST-paxillin fusion protein containing residues 54–191 and GST-paxillin fusion protein containing residues 168–313 of paxillin sequence were both observed to retain the primary phosphoserine-containing peptide (results not shown). These results demonstrated that both the primary site(s) of serine phosphorylation, as well as the serine kinase-binding site, reside within residues 168–191 of paxillin sequence.

A GST-paxillin fusion protein containing residues 168–191 was then synthesized and subjected to kinase assays *in vitro* as previously described. As shown in Figure 3(A), GST-paxillin 168–191 (lane 2) was highly phosphorylated in kinase assays, indicating that this fusion protein was efficiently precipitating one or more kinases. We did not, however, observe phosphorylation of either the p100 protein or FAK, as seen in assays performed with GST-paxillin 54–313 (Figure 3A, lane 1). Immunoblotting experiments confirmed that FAK was not precipitated by GST-paxillin 168–191 (results not shown). The absence of FAK binding to this fusion protein is consistent with our definition of the FAK-binding site on paxillin [30]. It has not yet been determined whether the p100 protein is absent from GST-paxillin 168–191 precipitates or is present in an unphosphorylated form. A phosphoamino acid analysis of phosphorylated GST-paxillin 168–191 (Figure 3B) showed that this

Figure 3 Serine kinase binding site lies within amino acids 168–191 of paxillin

(A) GST–paxillin 54–313 (lane 1) and GST–paxillin 168–191 (lane 2) were phosphorylated in *in vitro* kinase assays. (B) A phosphoamino acid analysis of GST–paxillin 168–191 indicated that the phosphorylation of this fusion protein was on serine residues only, consistent with the observation that GST–paxillin 168–191 precipitates a serine kinase but not FAK. Abbreviations: p100, 100 kDa protein; PS, phosphoserine; PT, phosphothreonine; PY, phosphotyrosine.

Figure 4 Identification of the major site(s) of serine phosphorylation as Ser-188 and/or Ser-190

To identify the principal site(s) of serine phosphorylation, site-directed mutagenesis was performed to substitute alanine residues for serine residues that fell within residues 168–191 of GST–paxillin 54–313. A double mutant, Ser-188/190 → Ala (B) demonstrated a 95% decrease in radiolabelling of the major phosphoserine-containing peptide compared with wild-type GST–paxillin 54–313 (A). The minor phosphopeptide migrating northwest of peptide 2 in the wild-type map (A, peptide a) was not consistently observed and was of variable intensity.

fusion protein was phosphorylated on serine, consistent with the hypothesis that residues 168–191 of paxillin are sufficient for the association of a serine kinase with paxillin.

Figure 5 Adhesion of fibroblasts to FN activates kinases that phosphorylate GST–paxillin 54–313 on sites similar to sites phosphorylated by smooth-muscle kinases

Lysates were prepared from CEFs seeded on either PL or FN. Kinases were precipitated from these lysates by GST–paxillin 54–313 as described in the legend to Figure 1. GST–paxillin 54–313 was phosphorylated *in vitro* by the co-precipitating kinases, and phosphorylated GST–paxillin 54–313 was subjected to two-dimensional phosphopeptide mapping.

The principal site of serine phosphorylation of paxillin *in vitro* is Ser-188 and/or Ser-190

Site-directed mutagenesis was used to substitute alanine residues for individual serine residues within amino acids 168–191 of GST–paxillin 54–313. Two-dimensional maps were then constructed from mutated fusion proteins phosphorylated *in vitro*. Surprisingly, none of these mutants demonstrated any significant decrease in radiolabelling of the major phosphoserine-containing peptide (results not shown). In contrast, a double mutant containing alanine substitutions for both Ser-188 and Ser-190 resulted in a more than 95% decrease in the phosphorylation of this peptide (Figure 4). These results suggest that both Ser-188 and Ser-190 can be efficiently phosphorylated by the co-precipitating serine kinase.

Cells grown on FN demonstrate enhanced phosphorylation of paxillin on both serine and tyrosine residues

Having defined the phosphorylation status of GST–paxillin 54–313 labelled *in vitro*, it was important to determine whether the sites phosphorylated *in vitro* were of physiological relevance. Because changes in paxillin phosphorylation have been studied most thoroughly in an integrin-mediated cell adhesion system, we evaluated the profile of paxillin phosphorylation elicited by such integrin activation. CEFs were seeded on either FN or an irrelevant substrate, PL. Lysates prepared from PL- or FN-plated cells were incubated with GST–paxillin 54–313 and kinase assays were performed *in vitro* as described above. Phosphopeptide maps constructed from GST–paxillin 54–313 radiolabelled by associated fibroblast kinases (Figure 5) demonstrated a similar pattern of phosphorylation to that previously observed with smooth-muscle kinases (Figure 2A). Notably, the phosphorylation of peptides that migrated to positions representing Tyr-118- and Ser-188/190-containing phosphopeptides was enhanced in FN-plated cells compared with PL-plated cells. This indicates that the activities of serine and tyrosine kinases that associate with paxillin *in vitro* are stimulated by cell adhesion.

To determine whether endogenous paxillin is also a target for this activity, the phosphorylation profile of native paxillin was characterized from FN- or PL-plated fibroblasts that were radiolabelled *in vivo* (see the Experimental section). Consistent with previous reports [15,17], paxillin precipitated from FN-plated cells demonstrated enhanced phosphorylation compared

Figure 6 Phosphorylation of paxillin is enhanced in cells plated on FN compared with cells plated on PL

(A) CEFs grown on either PL (lane 1) or FN (lane 2) were radiolabelled *in vivo* as described in the Experimental section. Radiolabelled cells were lysed and Čerenkov counting was used to normalize the lysates. Paxillin was precipitated with a monoclonal antibody, 165 [29]. The precipitates were resolved on SDS/PAGE gels and phosphorylated proteins were detected by autoradiography. The protein migrating at approx. 44 kDa is a proteolytic cleavage product of paxillin. (B) Paxillin was excised from the gel shown in (A) and subjected to phosphoamino acid analysis, which revealed an increase in both serine and tyrosine phosphorylation of paxillin derived from CEFs plated on FN compared with those plated on PL. Abbreviations: PS, phosphoserine; PY, phosphotyrosine.

with paxillin precipitated from PL-plated cells (Figure 6A). Phosphoamino acid analyses of phosphorylated paxillin revealed that spreading cells on FN effected a significant increase in the phosphoserine and phosphotyrosine content of paxillin. Of the total phosphorylation of paxillin observed in FN-plated cells (Figure 6B), 96% was due to phosphoserine; the remaining 4% of phosphorylation was contributed by phosphotyrosine. We also performed the phosphoamino acid analysis under different buffer conditions to confirm the absence of paxillin threonine phosphorylation (results not shown).

The phosphopeptide profile of paxillin labelled *in vivo* and precipitated from FN-plated cells (Figure 7B) was strikingly similar to the profile of GST-paxillin 54–313 labelled *in vitro* (Figures 5 and 7C). Peptide 1 contained phosphotyrosine and phosphoserine; peptides 2–5 contained phosphoserine only (results not shown). To evaluate whether the major peptides labelled *in vivo* co-migrated with peptides labelled *in vitro*, two-dimensional maps were constructed from mixed samples of paxillin immunoprecipitated from FN-plated cells and GST-paxillin 54–313 that had been labelled *in vitro* by smooth-muscle lysates (Figure 7D). Peptide 1 of endogenously labelled paxillin co-migrated with peptide 1 of GST-paxillin 54–313 labelled *in vitro*, and peptide 2 of paxillin labelled *in vivo* co-migrated with peptide 2 from GST-paxillin 54–313 labelled *in vitro*. As noted above, peptide 1 derived from paxillin labelled *in vivo* contained phosphoserine in addition to phosphotyrosine, whereas peptide 1 generated from GST-paxillin labelled *in vitro* contained phos-

Figure 7 Phosphopeptide mapping experiments on paxillin labelled *in vivo* suggest that the same sites that are phosphorylated *in vitro* are also phosphorylated in response to plating cells on FN

Radiolabelled paxillin immunoprecipitated from fibroblasts plated on either FN or PL was excised from SDS/polyacrylamide gels and subjected to tryptic phosphopeptide analyses [28]. In contrast with the minimal amount of labelling observed in paxillin precipitated from cells grown on PL (A), paxillin precipitated from FN-plated cells demonstrated phosphorylation at multiple sites (B). Phosphopeptide map of GST-paxillin 54–313 labelled *in vitro* (C). Phosphoamino acid analyses of these sites (results not shown) revealed that peptide 1 consisted of phosphotyrosine and phosphoserine, whereas peptides 2–5 contained phosphoserine only. To compare the sites of phosphorylation *in vitro* and *in vivo* more directly, samples of GST-paxillin labelled *in vitro* were mixed with samples of paxillin labelled *in vivo* and precipitated from FN-plated cells (D). Phosphopeptides representing the two major sites of phosphorylation *in vitro*, Tyr-118 (peptide 1) and Ser-188/190 (peptide 2), co-migrated with phosphopeptides generated from paxillin precipitated from FN-plated cells.

photyrosine only. The migration of the Tyr-118-containing peptide labelled *in vivo* was either not altered by the presence of phosphoserine residues in addition to phosphotyrosine residues or, alternatively, the phosphoserine signal was generated from a distinct peptide labelled *in vivo* that co-migrated with the Tyr-118-containing peptide.

Another major phosphoserine-containing peptide derived from paxillin precipitated from FN-plated cells, peptide 4 (Figure 7B), did not co-migrate with any peptides labelled *in vitro*. The phosphorylation of paxillin *in vivo* at this serine site (or sites) might reflect the activity of a kinase that is either not precipitated by GST-paxillin 54–313 or not active under the conditions used in our kinase assays *in vitro*. Alternatively the absence of phosphorylation at this site *in vitro* might result from the exclusion of this particular serine residue (or residues) from the amino acid sequence of GST-paxillin 54–313.

DISCUSSION

Previously we demonstrated that FAK binds with high affinity to a GST-paxillin fusion protein expressing residues 54–313 of paxillin and phosphorylates Tyr-118 [20,24]. In the present study we show that GST-paxillin 54–313 also associates with a serine kinase present in both fibroblast lysates and smooth-muscle extracts. We have mapped the serine kinase-binding site to a region of the paxillin molecule spanning residues 168–191. A GST-paxillin fusion protein containing residues 168–191 of paxillin precipitates, and is phosphorylated by, the serine kinase. Because this fusion protein does not bind FAK we conclude that the activity of the serine kinase, and also the association of the kinase with paxillin, are independent of FAK. Analyses of

GST-paxillin 54–313 phosphorylated *in vitro* indicate that the principal site of serine phosphorylation by the co-precipitating serine kinase is on Ser-188 and/or Ser-190.

To assess the physiological relevance of the sites phosphorylated *in vitro*, we performed experiments directed at characterizing the profile of paxillin phosphorylation *in vivo*. The results from these experiments revealed that plating cells on FN stimulates the phosphorylation of paxillin on both serine and tyrosine residues. Furthermore two of the peptides labelled *in vivo* co-migrated with peptides labelled *in vitro* corresponding to Ser-188/190 and Tyr-118, suggesting that these residues are most probably phosphorylated in response to integrin ligation to FN (Figure 7D). The observation that paxillin becomes phosphorylated on tyrosine in response to seeding cells on FN was expected, given other reports demonstrating the integrin-stimulated tyrosine phosphorylation of paxillin [15,17]. It is noteworthy, however, that the primary phosphotyrosine-containing peptide elicited by plating cells on FN is likely to represent the phosphorylation of Tyr-118, a residue that has been established as a target for phosphorylation by FAK. These results are consistent with numerous studies suggesting that adhesion-stimulated phosphorylation of paxillin is mediated in part by FAK.

Plating of fibroblasts on FN induced, in addition to tyrosine phosphorylation, a substantial increase in the phosphoserine content of paxillin. This finding implicates serine phosphorylation of paxillin as a putative mechanism for effecting adhesion-related signalling events. The potential phosphorylation of paxillin on serine residues has not been well studied, although one recent investigation of paxillin phosphorylation in macrophages yielded results that were similar to those reported here. DeNichilo and Yamada [31] found that growing macrophages on the integrin ligand, vitronectin, stimulated phosphorylation of paxillin composed of 99% phosphoserine and 1% phosphotyrosine. It was further suggested that the serine phosphorylation of paxillin observed in macrophages was mediated by PKC. In the present study we have not identified the serine kinase(s) responsible for the adhesion-stimulated phosphorylation of paxillin. Two major phosphoserine-containing peptides were generated from paxillin precipitated from fibroblasts labelled *in vivo* and plated on FN. One of these peptides (peptide 2, Figure 7B) most probably represents phosphorylated Ser-188/190. The amino acid sequence surrounding these residues does not represent a consensus site for any of the known kinases [32,33], so PKC is not likely to be directly responsible for phosphorylation at this site. However, several reports correlate increased PKC activity with paxillin phosphorylation. It is possible that PKC activity lies upstream in the signalling pathway connecting integrin activation to paxillin phosphorylation. Additionally, we currently have no information regarding either the identity of, or the consensus sequence surrounding, the second major site of paxillin serine phosphorylation (peptide 4, Figure 7B). Paxillin contains numerous consensus sequences for kinases such as PKC, protein kinase A, MAPK and p34^{cdc2} [24]. It is therefore plausible that multiple serine kinases, including PKC, might phosphorylate paxillin in response to integrin activation.

In addition to PKC [34–38], MAPK [39–41] and, more recently, p59ILK [42] have been identified as components of integrin-mediated signal transduction. Clearly, future investigations are needed to define better the kinases that directly phosphorylate focal adhesion proteins such as paxillin, in tandem with studies delineating how these kinases might interact in kinase cascades to signal adhesion-associated events including cytoskeletal reorganization and changes in gene transcription. Interest has centred predominantly on the role of tyrosine phosphorylation,

particularly because tyrosine phosphorylation is a well-characterized mechanism for facilitating protein–protein interactions. The formation of protein complexes is thought to promote multiple processes such as the recruitment of kinases to substrate, as well as the organization of cytoskeletal structures exemplified by focal adhesions and stress fibres. Interestingly, a recent study has shown that serine phosphorylation, as with tyrosine phosphorylation, can mediate protein–protein interactions [43]. It is intriguing to speculate that both serine and tyrosine phosphorylation of focal adhesion proteins such as paxillin are required for the correct formation of protein complexes involved in integrin-stimulated signalling pathways.

In summary, our results demonstrate that seeding fibroblasts on FN induces the phosphorylation of paxillin on both tyrosine and, more substantially, on serine residues. Although further studies will be needed to determine the identity of the serine kinase(s) responsible for paxillin phosphorylation, the phosphorylation of paxillin on serine residues in addition to tyrosine residues is consistent with the observation that cell adhesion stimulates both serine and tyrosine kinases. Future investigations directed at elucidating potential interactions between integrin-stimulated serine kinases and FAK, as well as defining the function of paxillin phosphorylation by these kinases, are likely to contribute significantly to our understanding of the fundamental mechanisms underlying cell adhesion.

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