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Rho stimulates tyrosine phosphorylation of focal adhesion kinase, p130 and paxillin

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SUMMARY

The small GTP-binding protein Rho rapidly stimulates the formation of focal adhesions and actin stress fibres when microinjected into serum-starved Swiss 3T3 fibroblasts. This response is inhibited by tyrosine kinase inhibitors. Addition of growth factors such as lysophosphatidic acid and bombesin to Swiss 3T3 cells stimulates a similar response, which is dependent on endogenous Rho proteins. To investigate signalling events regulated by Rho, we have scrape loaded Rho into serum-starved cells. Activated Rho stimulates the tyrosine phosphorylation of a number of proteins, including three proteins known to localise to focal adhesions, pp125^{FAK}, p130 and paxillin. Rho-induced phosphorylation of pp125^{FAK}, p130 and paxillin is observed in the absence of stress fibre formation and is, therefore, independent of Rho-induced actin polymerisation. We propose that the tyrosine kinase, pp125^{FAK}, and the putative adapter proteins, paxillin and p130, are components of a Rho-regulated signal transduction pathway, and that these protein tyrosine phosphorylation events are likely to be important for the regulation of focal adhesion formation.

Key words: Cytoskeleton, Focal adhesion kinase, Rho, Signal transduction, Tyrosine phosphorylation

INTRODUCTION

Cell-substratum adhesion plays a major role in a variety of cell functions. During the process of development and in the maintenance of tissue integrity, cells are required to adhere and interact with the extracellular matrix (ECM) in a tightly controlled manner. Conversely, during tumorigenesis and metastasis these interactions become deregulated. In fibroblasts, the intracellular actin cytoskeleton is involved in the control and maintenance of cell-substratum interactions and has an important role in cell motility. Actin stress fibres, for example, are transient, contractile bundles of actin filaments anchored to the plasma membrane at focal adhesions; sites where the cell contacts the substratum through transmembrane integrins (reviewed by Burridge et al., 1988). Contraction of anchored stress fibres allows the cell to exert tension on the substratum, a process that is important in wound healing and morphogenesis (reviewed by Horwitz and Thiery, 1994). In combination with other actin-based cytoskeletal structures, the contractile stress fibres may also be important in coordinating cell movement (Lee et al., 1993; Stossel, 1993). Rho, a Ras-related GTP-binding protein, rapidly stimulates the formation of actin stress fibres and focal adhesions when microinjected into a variety of cell types (Paterson et al., 1990; Ridley and Hall, 1992; Ridley et al., 1995). The Rho GTPase is an essential component of a signalling pathway linking extracellular factors to the formation of stress fibres and focal adhesions (Ridley and Hall, 1992). Two Rho-related GTPases, Rac and Cdc42Hs, also regulate growth factor-induced actin reorganisation. Rac is required for the rearrangement of the cortical actin network into membrane ruffles and lamellipodia (Ridley et al., 1992), while Cdc42Hs is required for the formation of filopodia (Kozma et al., 1995; Nobes and Hall, 1995). Analysis of the roles of Rho, Rac and Cdc42Hs in regulating cytoskeletal organisation suggests that cell-substratum adhesion and cell motility are controlled by interacting signal transduction cascades (Ridley et al., 1992; Kozma et al., 1995; Nobes and Hall, 1995).

The central role of Rho in regulating the formation of stress fibres and focal adhesions is well established, but the upstream and downstream components of this Rho-dependent signalling pathway have not been identified. Rho, like Ras, cycles between an active, GTP-bound state and an inactive, GDP-bound state. A number of proteins have been shown to regulate this cycling in vitro, and these are likely activators and/or targets of Rho (Boguski and McCormick, 1993; Ridley, 1995). However, the specific involvement of any of these proteins in the Rho signalling pathway in cells has not been definitively established.

Several lines of evidence suggest that protein tyrosine phosphorylation is important in the Rho signalling pathway. For example, stimulation of Swiss 3T3 fibroblasts with lysophosphatidic acid (LPA) or bombesin rapidly stimulates the Rho-dependent formation of stress fibres and focal adhesions and concomitantly induces tyrosine phosphorylation of a number of proteins, including focal adhesion kinase (pp125^{FAK}), p130 and the 68 kDa cytoskeletal protein, paxillin (Zachary et al., 1992, 1993; Sinnett-Smith et al., 1993; Chrzanowska-

Wodnicka and Burridge, 1994; Ridley and Hall, 1994; Seufferlein and Rozengurt, 1994). In addition, focal adhesions contain high levels of tyrosine phosphorylated proteins and tyrosine kinases such as pp125^{FAK} (Maher et al., 1985; Schaller et al., 1992). One approach, therefore, to analysing the Rho signal transduction pathway has been to use tyrosine kinase inhibitors. In this way, it has been demonstrated that a tyrosine kinase is required for LPA-induced activation of Rho (Nobes et al., 1995). Similarly, tyrosine kinase inhibitors can prevent Rho-induced formation of stress fibres, implying that a tyrosine kinase is required for signalling downstream of Rho (Ridley and Hall, 1994).

Another approach to investigating Rho-dependent signalling pathways has been to utilise C3 transferase (C3), an exoenzyme produced by *Clostridium botulinum*, that ADP-ribosylates and inhibits Rho protein (Aktories et al., 1989; Sekine et al., 1989; Paterson et al., 1990). Prolonged incubation of cells with C3 has been reported to inhibit the LPA and neuropeptide-induced tyrosine phosphorylation of pp125^{FAK} and paxillin (Kumagai et al., 1993; Rankin et al., 1994). In addition, C3 has been used to demonstrate a requirement for Rho in the activation of phosphatidylinositol 3-kinase (PI 3-kinase) and phosphatidylinositol 4-phosphate 5-kinase (PIP 5-kinase) (Kumagai et al., 1993; Zhang et al., 1993; Chong et al., 1994). Such experiments using C3 transferase show that Rho is necessary for these signalling events, but may only reflect an indirect requirement for Rho-induced cytoskeletal reorganisation.

Direct biochemical analysis of Rho-induced signals requires an efficient and reproducible method of introducing activated Rho protein into large numbers of cells. The technique of scrape loading allows the analysis of rapid and transient responses to exogenously added proteins. In addition, it allows the direct effects of a protein to be separated from possibly indirect changes observed in established cell lines. Scrape loading has been used successfully to dissect the Ras signalling pathway, providing evidence for the involvement of Ras in activation of protein kinase C (Morris et al., 1989), elevation of c-myc expression (Lloyd et al., 1989) and activation of MAP2 kinase (ERK2; Leevers and Marshall, 1992). By scrape loading, we have examined direct responses to Rho protein in quiescent Swiss 3T3 fibroblasts. Specifically, we have demonstrated that Rho induces changes in protein tyrosine phosphorylation and we have identified proteins that lie downstream of Rho in the signalling pathway regulating focal adhesion formation.

MATERIALS AND METHODS

Materials

The mouse anti-FAK antibody 2A7 and the anti-phosphotyrosine antibody 4G10 were obtained from Upstate Biotechnology Incorporated. The anti-paxillin antibody 165 was a gift from Christopher E. Turner. A monoclonal anti-paxillin antibody was also obtained from Transduction Laboratories. The anti-p130 antibody 4F4 was a gift from J. Thomas Parsons. Horseradish peroxidase-linked sheep anti-mouse immunoglobulin (Ig) and donkey anti-rabbit Ig were obtained from Amersham. Albumin-agarose, agarose-linked anti-mouse IgG, the mouse anti-phosphotyrosine antibody PT-66, LPA, bovine serum albumin (BSA), fluorescein isothiocyanate (FITC)-dextran, cytochalasin D, poly-L-lysine (PLL) and fibronectin (FN) were from Sigma Chemical Co.

Cell culture and scrape loading with recombinant proteins

Swiss 3T3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum. In preparation for scrape loading, cells were seeded on 100 mm dishes at a density of 8×10^5 per dish, grown to confluence for three days, then washed and incubated in serum-free DMEM for 16 hours. The cells were washed twice with phosphate buffered saline (PBS), recombinant protein was added in 160 μl of scrape loading (SL) buffer containing 10 mM Tris-HCl, pH 7.0, 114 mM KCl, 15 mM NaCl, 5.5 mM $MgCl_2$ (Leevers and Marshall, 1992) and cells were scrape loaded according to the method of Morris et al. (1989). V14 RhoA, V14A37 RhoA, V12 Rac1, V12 H-Ras and C3 transferase were expressed from the pGEX-2T vector as glutathione S-transferase fusion proteins (Smith and Johnson, 1988) and purified as previously described (Ridley and Hall, 1992). Active protein concentrations were determined by a filter binding assay using [^{3}H]GDP or [^{3}H]GTP as described (Hall and Self, 1986). Recombinant Rho, Rac and Ras proteins were scrape loaded at an active concentration of 19 μg/ml. The lowest concentration of C3 transferase required to block the V14 RhoA-induced response was titrated to 230 μg/ml total protein. BSA was scrape loaded at a concentration of 1 mg/ml.

LPA and cytochalasin D treatment of cells

For stimulation with LPA, cells were scrape loaded with SL buffer or with C3 transferase, as described above, washed into serum-free DMEM and incubated at 37°C, 10% CO_2 for 20 minutes. LPA was added to the cells at a concentration of 1 μg/ml for 10 minutes. The cells were then pelleted and lysed for analysis by immunoprecipitation. For cytochalasin D treatment, serum-starved cells were incubated in 0.1 μM, 0.65 μM or 1.2 μM cytochalasin D for 1 hour at 37°C, 10% CO_2 prior to scrape loading. The cells were either pelleted and lysed for analysis by immunoprecipitation, or were plated on coverslips coated with fibronectin (FN) for immunocytochemical analysis (see below).

Immunoprecipitation

Scrape loaded cells were washed into serum-free DMEM, incubated at 37°C, 10% CO_2 for various time points up to 1 hour and then pelleted by centrifugation (1,000 rpm for 5 minutes at 4°C). Cell pellets were lysed in 1 ml of ice-cold lysis buffer containing 10 mM Tris-HCl, pH 7.6, 5 mM EDTA, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM NaF, 1% Triton X-100, 100 μM Na_3VO_4 and 1 mM phenylmethylsulfonyl fluoride. Lysates were clarified by centrifugation at 15,000 ***g*** for 10 minutes and precleared by incubation with albumin-agarose for 1 hour at 4°C. Proteins were immunoprecipitated by incubation with antibody for 3 hours (2A7, and anti-paxillin antibodies) or 16 hours (4F4) at 4°C, followed by addition of goat anti-mouse IgG coupled to agarose (10 μl) and further incubation for 1 hour at 4°C. Immunoprecipitates were washed three times with lysis buffer, extracted in 2× SDS-PAGE sample buffer, then electrophoresed in 7.5% SDS-polyacrylamide gels and analysed by western blotting.

Western blotting

For analysis of whole cell lysates by western blotting, scrape loaded cells were washed and pelleted as described above. Pelleted cells were lysed in 250 μl of 1× SDS-PAGE sample buffer. Equal volumes of each lysate were electrophoresed on 7.5% SDS-polyacrylamide gels, and proteins were transferred from the gel to nitrocellulose membranes. Membranes were blocked in 5% non-fat dried milk in TBS (20 mM Tris-HCl, pH 7.6, 137 mM NaCl) containing 0.05% Tween-20, and then incubated with primary antibody diluted in TBS-Tween containing 1% non-fat dried milk, for 16 hours at 4°C (2A7, 1:1,000 dilution) or 2 hours at room temperature (anti-Rho, 1:15,000; 4G10 and PT-66, 1:10,000; anti-paxillin, 1:1,000). Membranes were then incubated for 1 hour at room temperature with a 1:15,000 dilution of a horseradish peroxidase-conjugated sheep anti-mouse or donkey

anti-rabbit antibody (Amersham), in TBS-Tween containing 1% non-fat dried milk. Immunoreactive bands were visualized by enhanced chemiluminescence (ECL) as described by the manufacturer (Amersham).

Immunofluorescence and microscopy

Sterile glass coverslips were placed in multiwell dishes, washed with distilled water and then incubated with either 13.3 μg/ml poly-L-lysine (PLL) for 10 minutes at room temperature, or 50 μg/ml FN for 90 minutes at 37°C. Cells were scrape loaded, washed into serum-free medium and added to coated coverslips at a density of 1×10^5 cells per ml. After incubation at 37°C, 10% CO_2 for time-points of 10 minutes up to 1 hour, cells were fixed in 3% paraformaldehyde and permeabilized in 0.2% Triton X-100. Actin filaments were detected as previously described with tetramethylrhodamine isothiocyanate (TRITC)-labelled phalloidin (Ridley and Hall, 1992). The formation of focal adhesions was investigated by staining cells with the anti-phosphotyrosine antibody PT-66, as previously described (Ridley and Hall, 1994). FN staining was carried out on scrape loaded cells incubated for 30 minutes, 37°C, 10% CO_2 on PLL coated coverslips. Cells were fixed and incubated with a 1:200 dilution of a rabbit anti-FN antibody (Dako Ltd) for 30 minutes at RT, followed by incubation with a 1:400 dilution of FITC-donkey anti-rabbit (Pierce) secondary antibody for 40 minutes, RT. Cells were viewed on a Zeiss Axiophot microscope and photographed with Kodak TMY-400 film.

RESULTS

Immunocytochemical analysis of scrape loaded cells

We have used the method of scrape loading to identify components of the Rho signal transduction pathway leading to the formation of actin stress fibres and focal adhesions in Swiss 3T3 fibroblasts. Scrape loading involves the physical removal of cells from their ECM, such that they become transiently permeable to molecules present in their surrounding solution (McNeil et al., 1984; Morris et al., 1989). We found that this method of introducing protein into cells was both efficient and reproducible. The percentage of permeable cells in the scraped cell population was estimated by scrape loading with fluorescein isothiocyanate (FITC)-dextran (McNeil et al., 1984). FITC-dextran of 20,000 kDa (approximately the same molecular mass as Rho) was loaded into cells at concentrations of 0.6 mg/ml (equivalent to the total protein concentration of scrape loaded Rho used in subsequent experiments) and 1.2 mg/ml. At both concentrations fluorescence was observed in over 80% of cells (data not shown). Dextran positive cells were uniformly fluorescent, although the level of fluorescence in each cell was variable.

The short term effects of scrape loading on cell morphology and actin cytoskeletal organisation were determined immunocytochemically. Quiescent, serum-starved Swiss 3T3 cells were scrape loaded with scrape loading (SL) buffer alone, or with the recombinant, constitutively activated form of Rho protein, Val 14 RhoA (V14 RhoA) (Paterson et al., 1990). Scraped cells were seeded in serum-free medium onto coverslips coated with poly-L-lysine (PLL), incubated for up to 1 hour and then fixed and stained with TRITC-phalloidin to visualise actin filaments.

A striking difference in the organisation of the actin cytoskeleton was observed between control and V14 RhoA-scraped cells seeded on PLL (Fig. 1A,B). Within 10 minutes the majority of cells had adhered to the PLL. Cells scrape loaded with buffer alone immediately started to spread out and by 30 minutes had a large, round, flattened appearance, with a concentration of actin filaments in lamellipodia at the leading edge of the cell membrane (Fig. 1A). In contrast, cells loaded with V14 RhoA had a more contracted morphology with long cables or spikes of polymerised actin extending radially from

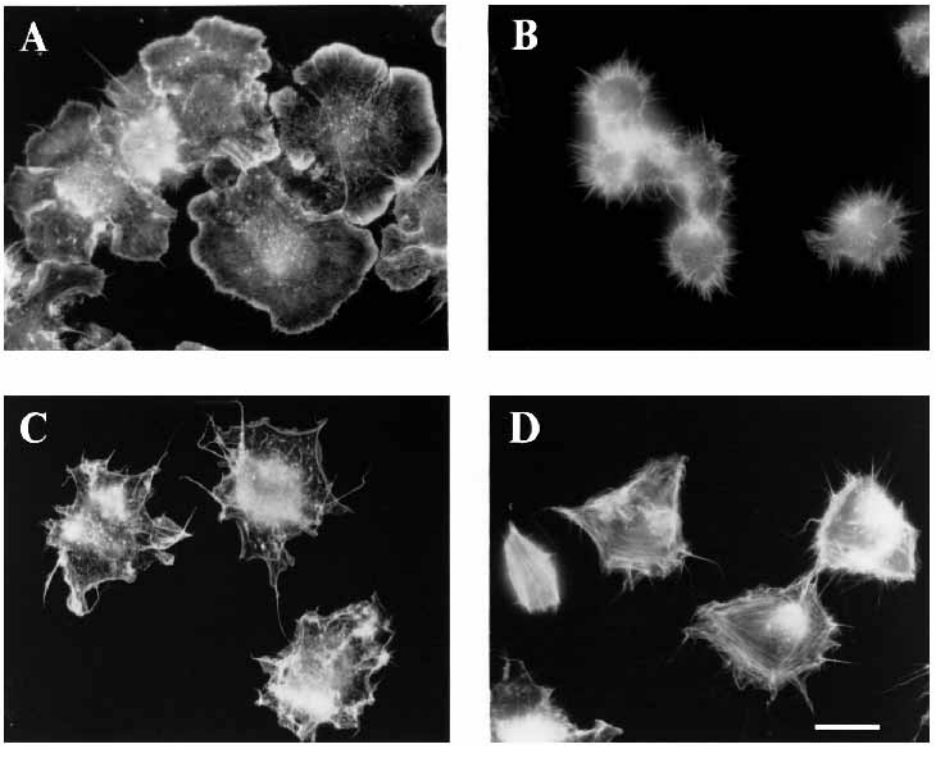

Fig. 1. Actin reorganisation in Swiss 3T3 cells scrape loaded with V14 RhoA. Quiescent serum-starved Swiss 3T3 cells were scrape loaded with SL buffer alone (A and C) or with 19 μg/ml V14 RhoA (B and D). Scraped cells were washed into serum-free medium and added to coverslips coated with poly-L-lysine (A and B) or fibronectin (C and D). After incubation for 30 minutes at 37°C, 10% CO_2, the cells were fixed, permeabilized and stained with TRITC-labelled phalloidin to visualise actin filaments. Bar, 20 μm.

the periphery of the nucleus (Fig. 1B). Differences in actin reorganisation between Rho scraped cells and control scraped cells were discernible within 15 minutes of plating on PLL (data not shown). By 30 minutes these morphological differences were much more pronounced, with an increase in the number and length of actin fibres extending from Rho scraped cells. In 3 independent experiments, we found that the number of cells showing a morphological response after scrape loading with Rho protein increased from an average of 58% at 15 minutes to a maximal level of 86% at 30 minutes; 150-250 cells were analysed at each time point (data not shown). The maximal number of responding cells was consistent with the percentage of cells taking up FITC-dextran (see above). No structures resembling focal adhesions were observed in Rho-scraped cells plated on PLL, as determined by staining with an anti-phosphotyrosine antibody (data not shown).

To examine Rho-induced cytoskeletal reorganisation in the presence of integrin interactions, scrape loaded cells were seeded onto coverslips coated with fibronectin (FN). Scrape loading with V14 RhoA stimulated the formation of actin stress fibres and focal adhesions in cells seeded on FN (Fig. 1D and data not shown). This response was indistinguishable from the response of serum-starved Swiss 3T3 cells to microinjected V14 RhoA (Ridley and Hall, 1992). A low level of stress fibre formation was observed in control cells scrape loaded with SL buffer (Fig. 1C), but in V14 RhoA-scraped cells the response was much faster and many more stress fibres were observed.

The differences in actin organisation between Rho-scraped cells plated on PLL and FN suggest that the manifestation of the Rho response is influenced by the ECM. It is interesting to note that, as a result of the scraping procedure, scrape loaded cells retained residual amounts of FN on their surface (data not shown). Pre-treatment of cells for 2 hours in 25 μg/ml cycloheximide to prevent de novo synthesis of FN did not reduce the amount of FN observed on the scraped cells, and had no effect on the morphology of cells plated on PLL (data not shown). Given the differences in actin organisation between scraped cells plated on PLL and FN, it is unlikely that the FN present on the surface of the scrape loaded cells contributed significantly to the morphological response.

To confirm that the observed reorganisation of the actin cytoskeleton was specific to addition of V14 RhoA, cells were scrape loaded with equivalent amounts of two related proteins, Ras (V12 H-Ras) and Rac (V12 Rac1), and plated on both PLL and FN. The morphology of cells loaded with either of these two proteins was not significantly different from that of cells scraped with SL buffer alone, although cells loaded with V12 Rac1 did appear to spread more rapidly on FN (data not shown). Microinjection of serum-starved Swiss 3T3 cells with V12 Rac1 or V12 H-Ras induces the reorganisation of actin into lamellipodia and membrane ruffles (Ridley et al., 1992); however, scrape loaded cells spreading on PLL or FN have multiple lamellipodia (Fig. 1). This suggests that in spreading cells endogenous Rac is already activated, and that addition of activated exogenous Rac has little further effect.

Rho induces protein tyrosine phosphorylation

Immunocytochemical analysis confirmed that scrape loading could provide an efficient and reproducible method of analysing the Rho pathway at a biochemical level. Given the evidence that a tyrosine kinase acts downstream of Rho in the signal transduction pathway leading to cytoskeletal reorganisation (Ridley and Hall, 1994), we examined whether Rho would induce changes in protein tyrosine phosphorylation. Lysates of scrape loaded cells were separated by SDS-PAGE for analysis by western blotting, and blots were probed with an anti-phosphotyrosine antibody. The overall level of protein tyrosine phosphorylation in cells was immediately reduced upon scrape loading, presumably due to the loss of cell-ECM interactions (see Burridge et al., 1992). For example, the major phosphotyrosine-containing protein of approximately 125 kDa observed in lysates from adherent serum-starved cells was dephosphorylated in cells lysed immediately after scraping (Fig. 2A, compare lanes 1 and 4). This reduced level of background phosphorylation in scrape loaded cells facilitated analysis of later responses to protein addition.

An increase in the phosphotyrosine content of a number of proteins was observed 30 minutes after cells were scrape loaded with V14 RhoA. In particular, V14 RhoA stimulated a major increase in phosphorylation of a diffuse band of proteins in the molecular mass range of 120-140 kDa (Fig. 2A, lane 3). No such response was observed in control cells scraped with bovine serum albumin (BSA) or with a constitutively activated mutant of Rac, V12 Rac1 (Fig. 2A, lanes 5 and 6). Equal loading of proteins was determined by Coomassie Blue staining of the gel following western blotting (Fig. 2B). The response induced by V14 RhoA was not observed in cells incubated for 15 minutes (Fig. 2A, lane 2), but was sustained for 1 hour after scrape loading (data not shown). As we were examining direct, early responses to Rho addition, time-points later than 1 hour were not tested.

The concentration of V14 RhoA required to induce significant phosphorylation of the 120-140 kDa proteins (Fig. 2A and data not shown) was titrated to 19 μg/ml and this concentration was therefore used in all subsequent experiments.

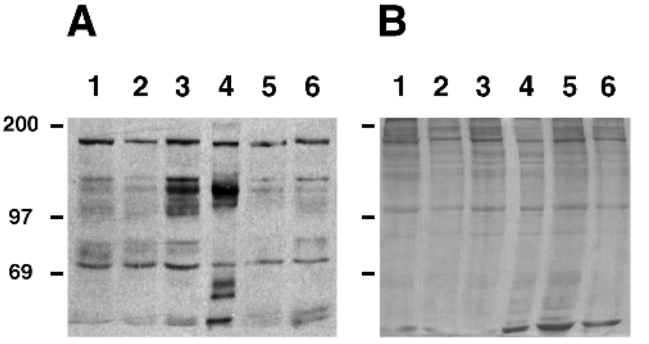

Fig. 2. Scrape loading with Rho stimulates protein tyrosine phosphorylation. (A) Cells were scraped with V14 RhoA and then lysed immediately (lane 1), or incubated for 15 minutes (lane 2) or 30 minutes (lane 3) in serum-free medium prior to lysis. As controls, a lysate was made from adherent, quiescent cells (lane 4) and from cells incubated for 30 minutes after scraping with BSA (lane 5) or V12 Rac1 (lane 6). Equal aliquots of the cell lysates were separated by SDS-PAGE and blotted with the anti-phosphotyrosine antibody 4G10. The molecular mass markers (in kilodaltons) are indicated on the left of the blot. (B) Coomassie stained gel of proteins remaining after western transfer of the blot in (A). Molecular mass markers corresponding to those in (A) are indicated by the lines to the left of the gel.

Rho stimulates tyrosine phosphorylation of pp125^{FAK}

The proteins phosphorylated in response to Rho are likely components and/or targets of a Rho-dependent signal transduction pathway. We therefore set out to identify these tyrosine phosphorylated proteins. One candidate for a downstream target of Rho was pp125^{FAK}, the 125 kDa tyrosine kinase that localises to focal adhesions (Hanks et al., 1992; Schaller et al., 1992). In order to examine pp125^{FAK} phosphorylation in response to Rho, we used the anti-pp125^{FAK} antibody 2A7 to immunoprecipitate the protein from lysates of scrape loaded cells. Immune complexes were then separated by SDS-PAGE and western blotted with the anti-phosphotyrosine antibody 4G10. An increase in tyrosine phosphorylation of pp125^{FAK} was observed 30 minutes after scrape loading with V14 RhoA (Fig. 3A, lanes 1 and 2). This Rho-induced pp125^{FAK} phosphorylation was equivalent to that observed in mock scrape loaded cells stimulated with LPA prior to lysis (Fig. 3A, lane 4). LPA stimulation provided a control for the level of the Rho-induced response. In addition, it demonstrated that scraped cells were able to respond to extracellular stimuli leading to protein tyrosine phosphorylation, even in the absence of cell-substratum interactions.

To demonstrate that the increase in pp125^{FAK} phosphorylation was due to Rho and not to a contaminant in the Rho preparations, V14 RhoA was scrape loaded in combination with the Rho inhibitor, C3 transferase. In these cells there was no increase in phosphorylation of pp125^{FAK} (Fig. 3A, lane 3).

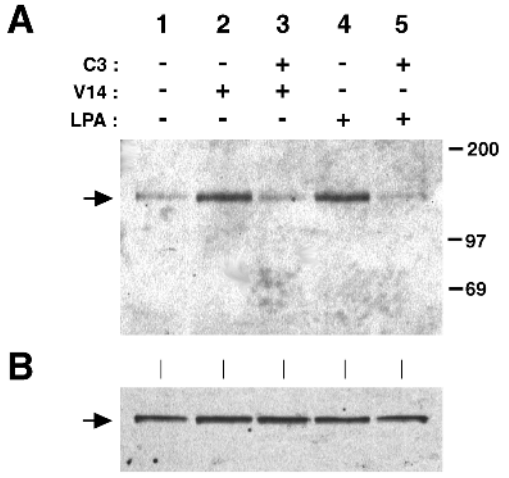

Fig. 3. Rho and LPA induce equivalent levels of pp125^{FAK} tyrosine phosphorylation. (A) Quiescent serum-starved Swiss 3T3 cells were scraped with buffer alone (lanes 1 and 4), V14 RhoA (lane 2), V14 RhoA and C3 transferase together (lane 3) or C3 transferase alone (lane 5). Samples in lanes 1-3 were incubated for 30 minutes in serum-free medium prior to lysis. Samples in lanes 4 and 5 were incubated for 20 minutes and then stimulated with LPA (1 μg/ml) for 10 minutes prior to lysis. All samples were subsequently immunoprecipitated with the anti-pp125^{FAK} antibody 2A7. Immune complexes were separated by SDS-PAGE and western blotted with the anti-phosphotyrosine antibody 4G10. Crosses above the blot indicate addition of C3 transferase (C3), V14 RhoA (V14) and LPA to the cells. Molecular mass markers are on the right of the blot. The position of pp125^{FAK} is indicated by the arrow on the left of the blot. (B) The blot in (A) was reprobed with 2A7 to show equal loading of pp125^{FAK} in all lanes. Vertical lines correspond to lanes 1-5 in (A). The position of pp125^{FAK} is indicated by the arrow on the left.

Similarly, LPA-induced pp125^{FAK} phosphorylation was blocked in cells scrape loaded with C3 transferase (Fig. 3A, lane 5), confirming that LPA-induced pp125^{FAK} phosphorylation is a Rho-dependent response. Equivalent loading of pp125^{FAK} in these experiments was determined by reprobing the blots with an anti-pp125^{FAK} antibody (Fig. 3B).

To confirm further the specificity of the response to Rho, we scrape loaded cells with equivalent amounts of a recombinant inactive mutant of Rho, V14A37 RhoA (Paterson et al., 1990), and with V12 Rac1 (Fig. 4). Phosphorylation of pp125^{FAK} was only observed in those cells scrape loaded with recombinant active Rho protein (Fig. 4A, FAK IP, lane 2). Blots were reprobed with an anti-pp125^{FAK} antibody to demonstrate equal loading between samples (Fig. 4B, FAK). From these results we conclude that Rho stimulates an increase in tyrosine phosphorylation of pp125^{FAK} equivalent to that induced by stimulation of cells with LPA.

Rho induces phosphorylation of p130 and paxillin

As described above, a diffuse band of proteins in the 120-140 kDa molecular mass range was phosphorylated in Rho-scraped cells (Fig. 2). Our results suggest that one component of this band is pp125^{FAK}. Another candidate molecule is a 130 kDa protein (p130) which, like pp125^{FAK}, was identified as a pp60$^{v\text{-}src}$ (v-Src) substrate in RSV-transformed chick embryo fibroblasts (Reynolds et al., 1989; Guan and Shalloway, 1992). Both pp125^{FAK} and p130 are phosphorylated in response to LPA (Seufferlein and Rozengurt, 1994; H. M. Flinn and A. J. Ridley, unpublished). We therefore examined p130 phosphorylation in response to scrape loading with V14 RhoA.

The anti-p130 antibody 4F4 (a gift from J. Thomas Parsons) was used to immunoprecipitate p130 from scraped cells, 30 minutes after protein addition. As with pp125^{FAK}, an increase in phosphorylation of p130 was observed in cells scrape loaded with the recombinant activated form of Rho, V14 RhoA (Fig. 4A, p130 IP, lane 2), but not in cells loaded with V14A37 RhoA or V12 Rac1 (Fig. 4A, p130 IP, lanes 3 and 4). Scrape loading of C3 transferase in combination with V14 RhoA completely blocked phosphorylation of p130 (data not shown). Equivalent loading of p130 in anti-phosphotyrosine blots could not be shown directly, as the anti-p130 antibody worked inefficiently on western blots. However, the experiments were carried out several times with consistent results and examination of co-precipitating proteins satisfied us that the lysates contained equal amounts of protein.

Another potential downstream target of Rho is paxillin, a 68 kDa cytoskeletal protein that co-localises with pp125^{FAK} in focal adhesions (Turner et al., 1990). Stimuli that phosphorylate pp125^{FAK} have also been shown to phosphorylate paxillin (Burridge et al., 1992; Zachary et al., 1993; Rankin et al., 1994; Seufferlein and Rozengurt, 1994) and the protein acts as a substrate for pp125^{FAK} (Turner, 1994; Schaller and Parsons, 1995). Long exposures of immunoblots from whole cell lysates showed that scrape loading with activated Rho protein induced increased tyrosine phosphorylation of proteins in the 60-70 kDa molecular mass range (data not shown). The monoclonal antibody 165 (a gift from Christopher E. Turner) was used to immunoprecipitate paxillin from cells scrape loaded with V14 RhoA, V14A37 RhoA and V12 Rac1. Anti-phosphotyrosine western blots demonstrated that there was an increase in paxillin tyrosine phosphorylation in response to

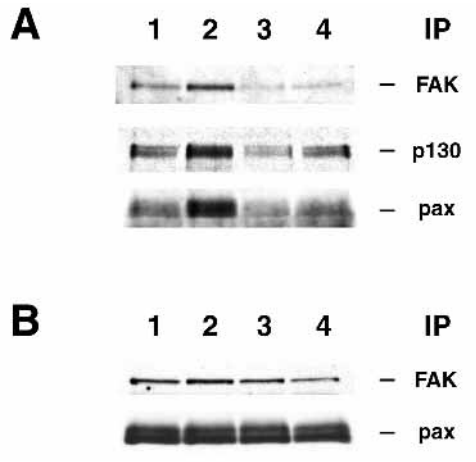

Fig. 4. Rho induces tyrosine phosphorylation of pp125^{FAK}, p130 and paxillin. (A) Alignment of anti-phosphotyrosine western blots of proteins immunoprecipitated from scraped cell lysates. Cells were scraped with buffer alone (lane 1), V14 RhoA (lane 2), V14A37 RhoA (lane 3) or V12 Rac1 (lane 4). Scraped cells were incubated for 30 minutes prior to lysis and immunoprecipitation. The antibodies used for immunoprecipitation (IP) are indicated to the right of each blot: 2A7 (FAK), anti-p130 antibody 4F4 (p130) and anti-paxillin antibody (pax). (B) FAK and pax blots from (A) were reprobed with 2A7 (FAK) and the anti-paxillin antibody (pax), respectively, to show equivalent protein loading. Blots are aligned as in (A).

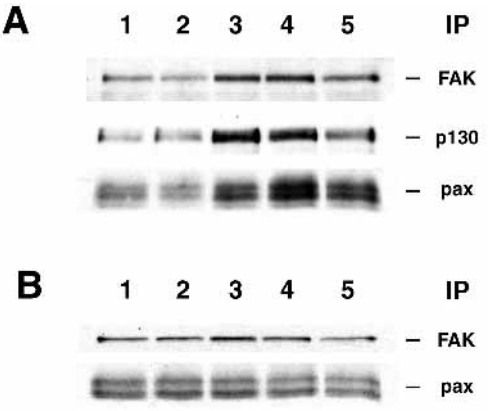

Fig. 5. Time-course of Rho-induced pp125^{FAK}, p130 and paxillin tyrosine phosphorylation. (A) Alignment of anti-phosphotyrosine western blots of proteins immunoprecipitated from scraped cell lysates. Cells were scraped with buffer alone (lane 1) or with V14 RhoA (lanes 2-5). Scraped cells were incubated for various lengths of time prior to lysis and immunoprecipitation: 15 minutes (lane 2), 30 minutes (lane 3), 45 minutes (lane 4) or 1 hour (lane 5). The antibodies used for immunoprecipitation (IP) are indicated to the right of each blot: 2A7 (FAK), 4F4 (p130) and anti-paxillin antibody (pax). (B) FAK and pax blots from A were reprobed with 2A7 (FAK) and the anti-paxillin antibody (pax), respectively, to show equivalent protein loading. Blots are aligned as in A.

V14 RhoA (Fig. 4A, pax IP, lane 2). The level of phosphorylation observed in cells scraped with V14A37 RhoA and V12 Rac1 was no higher than that obtained by scraping with buffer alone (Fig. 4A, pax IP, lanes 1, 3 and 4). Equivalent protein loading between samples was confirmed by re-probing the blot with a commercially obtained anti-paxillin antibody (Fig. 4B, pax IP).

Time-course of Rho-induced phosphorylation

We have demonstrated that tyrosine phosphorylation of pp125^{FAK}, p130 and paxillin is increased 30 minutes after cells are scrape loaded with activated Rho protein. To determine the time course of Rho-induced phosphorylation for each of the three proteins, cells were incubated for between 15 minutes to one hour after scrape loading with V14 RhoA. Tyrosine phosphorylation of all three proteins was transient, decreasing 1 hour after protein addition (Fig. 5A, lanes 1 to 5). Phosphorylation of pp125^{FAK} and p130 followed the same time course, reaching a peak at 30 minutes and remaining at that level up to 45 minutes after addition of Rho protein (Fig. 5A, FAK and p130 IP). An increase in phosphorylation of paxillin was also observed after 30 minutes, but the response reached its peak 45 minutes after protein addition (Fig. 5A, pax IP). This lag in phosphorylation of paxillin relative to pp125^{FAK} is consistent with observations suggesting that paxillin acts as a substrate of pp125^{FAK} (Turner, 1994; Schaller and Parsons, 1995).

Rho-induced phosphorylation is separable from Rho-induced stress fibre formation

A significant increase in tyrosine phosphorylation of pp125^{FAK}, p130 and paxillin was observed 30 minutes after scrape loading with V14 RhoA (Fig. 5). Similarly, Rho-induced changes in the actin cytoskeleton, although discernable within 15 minutes of scrape loading, increased in intensity over time reaching a maximal level by 30 minutes (Fig. 1 and data not shown). Taken together these results suggested that Rho-induced protein tyrosine phosphorylation may be dependent upon cytoskeletal reorganisation. To test this, cells were pre-treated with the actin filament disrupting agent, cytochalasin D, prior to scrape loading with V14 RhoA. Protein tyrosine phosphorylation in the cytochalasin D treated cells was then analysed and compared to actin stress fibre formation in cells plated on FN (Fig. 6; Table 1).

At the lowest concentration of cytochalasin D used (0.1 μM) the number of V14 RhoA scraped cells with actin stress fibres

Table 1. Effects of cytochalasin D on Rho-induced stress fibre formation and Rho-induced tyrosine phosphorylation

Scrape loaded cells	Cytochalasin D μM	% cells with actin stress fibres	Protein tyrosine phosphorylation
SL buffer	–	5	–
V14 RhoA	–	82	+
V14 RhoA	0.1	21	+
V14 RhoA	0.65	7	+
V14 RhoA	1.2	3	–

Serum-starved cells were scrape loaded with SL buffer or with V14 RhoA as normal, or pre-treated with increasing concentrations of cytochalasin D for 1 hour prior to scrape loading with V14 RhoA. The percentage of scraped cells containing actin stress fibres 30 minutes after plating on FN is shown. An average of 500 cells were counted per point. For comparison, the effect of cytochalasin D on protein tyrosine phosphorylation 30 minutes after scrape loading is also shown; these results are representative of 5 independent experiments. Increased tyrosine phosphorylation of pp125^{FAK}, p130 and paxillin above background is denoted by '+'. Background phosphorylation is denoted by '–'.

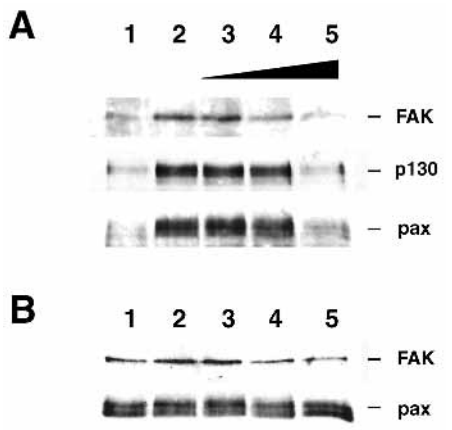

Fig. 6. Effect of cytochalasin D on Rho-induced protein tyrosine phosphorylation. (A) Alignment of anti-phosphotyrosine western blots of proteins immunoprecipitated from scraped cell lysates. Cells were scraped with buffer alone (lane 1) or with V14 RhoA (lanes 2-5), and were incubated for 30 minutes prior to lysis and immunoprecipitation. Cells in lanes 3-5 were pre-treated with increasing concentrations of cytochalasin D for 1 hour prior to scrape loading: 0.1 μM (lane 3), 0.65 μM (lane 4) and 1.2 μM (lane 5). The antibodies used for immunoprecipitation (IP) are indicated to the right of each blot: 2A7 (FAK), 4F4 (p130) and anti-paxillin antibody (pax). (B) FAK and pax blots from (A) were reprobed with 2A7 (FAK) and the anti-paxillin antibody (pax), respectively, to show protein loading. Blots are aligned as in (A).

was reduced from 82% to 21%, 30 minutes after scrape loading (Table 1). Despite the reduction in stress fibre formation, the cortical actin filament network in these cells still appeared intact (data not shown). Disruption of the actin cytoskeleton was more obvious in those cells incubated in 0.65 μM cytochalasin D. Only 7% of these cells contained actin stress fibres (Table 1), cell spreading was restricted and actin filaments accumulated in aggregates throughout the cytoplasm. The cytoskeleton of cells incubated in 1.2 μM cytochalasin D was more severely disrupted and only 3% of these cells contained any stress fibres (Table 1).

Rho-induced tyrosine phosphorylation of pp125^{FAK}, p130 and paxillin was unaffected in cells incubated with 0.1 μM and 0.65 μM cytochalasin D, conditions in which actin stress fibre formation was significantly reduced (Fig. 6; Table 1). Only in cells treated with 1.2 μM cytochalasin D, when the actin cytoskeleton was severely disrupted, was phosphorylation of these proteins reduced to a background level (Fig. 6). From these experiments we conclude that Rho-induced phosphorylation of pp125^{FAK}, p130 and paxillin is not dependent upon Rho-induced actin reorganisation. However, the presence of an intact cytoskeleton would appear to be necessary for phosphorylation of these proteins to occur.

DISCUSSION

The introduction of Rho proteins into fibroblasts by microinjection rapidly induces the formation of actin stress fibres and focal adhesions (Paterson et al., 1990; Ridley et al., 1992). These responses have previously been shown to be inhibited by tyrosine kinase inhibitors (Ridley and Hall, 1994), suggesting that protein tyrosine phosphorylation is required downstream of Rho in this signalling pathway. We now show that Rho protein stimulates the tyrosine phosphorylation of a number of proteins in serum-starved Swiss 3T3 cells, including three proteins known to localise to focal adhesions, pp125^{FAK}, paxillin and p130 (Turner et al., 1990; Schaller et al., 1992; Petch et al., 1995). This response is specific to Rho and is not induced by the related protein, Rac. In addition, introduction of C3 transferase, an inhibitor of Rho function, into these cells prevents LPA- and Rho-induced tyrosine phosphorylation of these proteins. These results indicate that Rho is both necessary and sufficient for tyrosine phosphorylation of pp125^{FAK}, paxillin and p130.

In examining the time-course of Rho-induced phosphorylation, we found that phosphorylation of pp125^{FAK} and p130 increased 30 minutes after scrape loading cells with Rho. Phosphorylation of paxillin was also observed after 30 minutes, but peaked at 45 minutes, consistent with the view that paxillin is a substrate of pp125^{FAK} (Turner, 1994; Schaller and Parsons, 1995). Rho-induced changes in the actin cytoskeleton, although discernible within 15 minutes of scrape loading, reached a maximal level by 30 minutes. To test whether the observed protein tyrosine phosphorylation was dependent upon the changes in actin organisation, we examined Rho-induced phosphorylation in cells treated with the actin filament disrupting agent, cytochalasin D. Tyrosine phosphorylation of pp125^{FAK}, p130 and paxillin in response to Rho was observed under conditions in which actin stress fibre formation was almost completely inhibited. These results demonstrate that the Rho-induced signalling pathway leading to the phosphorylation of focal adhesion-associated proteins is independent of the Rho-induced pathway to actin reorganisation. Our conclusions are, therefore, in agreement with a previous report where Rho-induced formation of focal adhesions and polymerisation of actin were also shown to be separable events (Nobes and Hall, 1995).

Paxillin, pp125^{FAK} and p130 are components of focal adhesions (Turner et al., 1990; Schaller et al., 1992; Petch et al., 1995), the discrete integrin-associated protein complexes through which fibroblasts attach to the ECM. It has recently been shown that pp125^{FAK} is not required for the formation of focal adhesions, but may be involved in regulating turnover of these protein complexes during cell migration (Ilic et al., 1995). The sequences of paxillin and p130 suggest that they may function as adapter proteins involved in multiple protein-protein interactions. Paxillin, for example, contains putative SH2 and SH3 domain binding motifs and multiple LIM domains (Turner and Miller, 1994). A candidate p130, p130^{CAS}, possesses an SH3 domain together with several putative SH2-binding sites (Sakai et al., 1994). Therefore, although these proteins have no enzymatic activity, they are likely to play an important role in the regulation or formation of focal adhesions.

Although the precise roles of pp125^{FAK}, p130 and paxillin in focal adhesion formation have yet to be established, all three of the proteins have been implicated in signalling pathways which regulate cytoskeletal reorganisation. Cell adhesion to ECM for example, promotes tyrosine phosphorylation of pp125^{FAK}, paxillin and p130 (Burridge et al., 1992; Petch et al., 1995). The proteins are also phosphorylated on tyrosine in response to LPA and neuropeptides, including bombesin

(Zachary et al., 1992; Seufferlein and Rozengurt, 1994; H. M. Flinn and A. J. Ridley, unpublished observations), factors which signal through Rho to induce actin reorganisation (Ridley and Hall, 1992). In addition, prolonged treatment of Swiss 3T3 fibroblasts with C3 transferase blocks bombesin- and endothelin-induced tyrosine phosphorylation of pp125^{FAK} and paxillin, implicating Rho in the signalling pathway initiated by these neuropeptides (Rankin et al., 1994). Our experiments have provided direct evidence that pp125^{FAK}, paxillin and p130 are tyrosine phosphorylated in response to Rho. This Rho-induced phosphorylation occurs while the cells are in suspension, indicating that integrin-ECM interactions are not essential for the response. Therefore, although cell adhesion to ECM itself leads to phosphorylation of pp125^{FAK}, p130 and paxillin, Rho may transduce this response such that the addition of activated Rho bypasses the requirement for integrins. However, integrin-mediated interactions appear to be necessary for the establishment of Rho-induced actin stress fibres, as no stress fibres were observed in Rho-scraped cells seeded on PLL. Our results suggest that Rho stimulates the formation of contractile actin filaments, independent of integrin engagement. In fibroblasts plated on PLL, or in cell types which do not normally form stress fibres (e.g. neuronal cells, see Jalink et al., 1994), these filaments induce cell rounding and contraction. In fibroblasts plated on FN, integrin engagement leads to the organisation of the newly formed filaments into stress fibres linked to focal adhesions.

All three of the proteins we have identified as downstream targets of Rho are v-Src-associated substrates (Glenney and Zokas, 1989; Reynolds et al., 1989; Kanner et al., 1990, 1991; Guan and Shalloway, 1992; Weng et al., 1993). Paxillin and p130^{CAS} have also been found in association with the Src family kinase, v-Crk (Birge et al., 1993; Sakai et al., 1994). Tyrosine phosphorylation of paxillin mediated by pp125^{FAK} creates binding sites for Crk, Src and Csk, a Src-family negative regulatory kinase (Schaller and Parsons, 1995). Over-expressed Csk has independently been shown to localise to focal adhesions, possibly through interaction of the Csk SH2 domains with pp125^{FAK} and paxillin (Bergman et al., 1995). Taken together, these data suggest that there is considerable cross-talk between signalling pathways regulated by Rho and by Src family kinases.

Tyrosine phosphorylation of pp125^{FAK} in response to either cell adhesion or Src transformation, correlates with an increase in pp125^{FAK} kinase activity (Guan and Shalloway, 1992). Autophosphorylation of pp125^{FAK} on tyrosine 397 is essential for pp125^{FAK}-dependent phosphorylation of paxillin in vivo (Schaller and Parsons, 1995), but does not regulate in vitro kinase activity of pp125^{FAK} (Schaller et al., 1994). It has been proposed, therefore, that autophosphorylation of pp125^{FAK} in vivo permits binding and activation of a member of the Src family kinases, which further phosphorylates and activates pp125^{FAK} (Schaller et al., 1994; Calalb et al., 1995; Schaller and Parsons, 1995). The mechanism by which pp125^{FAK} is initially autophosphorylated is unknown. In vitro assays have demonstrated that both pp125^{FAK} and paxillin can bind to the cytoplasmic domain of the β1 integrin (Schaller et al., 1995). Therefore, it is possible that pp125^{FAK} association and subsequent autophosphorylation may occur via integrin clustering. We have shown that pp125^{FAK} is phosphorylated in response to Rho. Activated Rho, or a Rho effector must therefore stimulate pp125^{FAK} autophosphorylation. This might occur through a direct interaction or, alternatively, Rho might stimulate pp125^{FAK} autophosphorylation indirectly by initiating the clustering of integrins. Subsequent phosphorylation of adapter proteins such as p130 and paxillin would then influence their interaction with structural and/or regulatory components of focal adhesions.

We propose, therefore, that Rho-induced tyrosine phosphorylation of pp125^{FAK}, p130 and paxillin is required for the formation or turnover of focal adhesions. This pathway is separable from that of Rho-induced actin polymerisation, which may proceed through the Rho-dependent stimulation of phosphatidylinositol 4-phosphate 5-kinase activity to regulate PIP_2 levels (Stossel, 1993; Chong et al., 1994). A molecular link between actin filaments and Rho has recently been suggested by the cloning of a novel type of myosin, myr 5, with in vitro GAP activity for Rho-family GTPases (Reinhard et al., 1995). As myosins interact directly with the actin cytoskeleton, it is tempting to speculate that myr 5-like proteins, together with PIP_2, might act downstream of Rho in the pathway toward actin polymerisation, while pp125^{FAK}, paxillin and p130 are downstream of Rho in the pathway toward focal adhesion formation. By interacting with different effector molecules, Rho would thereby coordinate these cellular responses.

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