pp125\textsuperscript{FAK} tyrosine kinase activity is not required for the assembly of F-actin stress fibres and focal adhesions in cultured mouse aortic smooth muscle cells

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SUMMARY

The observed increase in phosphotyrosine content of focal adhesion-associated proteins, in response to integrin engagement, indicates a role for integrin-regulatable tyrosine kinase(s) in cytoskeletal re-organisation. The tyrosine kinase pp125\textsuperscript{FAK}, by virtue of its focal adhesion localisation in fibroblasts, represents a prime candidate to perform this function. We have investigated whether pp125\textsuperscript{FAK} performs a similar function in mouse aortic smooth muscle cells (MASMC). MASMC cultured for 16 hours exhibit F-actin stress fibres and focal adhesions. We have shown that vinculin, pp125\textsuperscript{FAK} and tyrosine-phosphorylated proteins are localised in focal adhesions during this time period. MASMC, under these culture conditions exhibit elevated pp125\textsuperscript{FAK} tyrosine kinase activity, as measured by an increased autophosphorylation potential. We investigated the development of F-actin stress fibres and focal adhesions in MASMC in response to adherence to fibronectin, conditions shown to promote cytoskeletal reorganisation in fibroblasts. Within 30 minutes, MASMC exhibited well-developed F-actin stress fibres and prominent focal adhesions which immunostained intensely for vinculin, pp125\textsuperscript{FAK} and phosphotyrosine. Adherence to fibronectin has been reported to activate pp125\textsuperscript{FAK} tyrosine kinase in fibroblasts, leading to the proposal that pp125\textsuperscript{FAK} plays a critical role in focal adhesion formation. Therefore pp125\textsuperscript{FAK} activation, in response to adherence to fibronectin, was investigated in MASMC. Anti-phosphotyrosine immunoblotting and in vitro kinase assays of MASMC lysates have revealed that, under conditions which promote focal adhesion formation, pp125\textsuperscript{FAK} remains inactive. Since overnight cultures of MASMC exhibited elevated pp125\textsuperscript{FAK} tyrosine kinase activity, we investigated whether these cells deposit their own combination of extracellular matrix (ECM) molecules and/or secrete factors into their conditioned medium which are capable of activating pp125\textsuperscript{FAK} tyrosine kinase. Our results indicate that MASMC-elaborated ECM, but not their conditioned medium, supported pp125\textsuperscript{FAK} tyrosine kinase activation. Furthermore, MASMC exposed to MASMC-ECM displayed a poorly defined F-actin stress fibre network and rudimentary focal adhesions. Thus we have demonstrated the existence of two adhesion-mediated situations in MASMC; one in which fibronectin promotes cytoskeletal reorganisation in the absence of pp125\textsuperscript{FAK} tyrosine kinase activity and the other in which cells adhering to MASMC-ECM display elevated pp125\textsuperscript{FAK} tyrosine kinase activity in association with an impaired ability to promote F-actin stress fibre and focal adhesion formation. These results indicate that in MASMC, pp125\textsuperscript{FAK} tyrosine kinase activity is not involved in F-actin stress fibre assembly and focal adhesion formation.

Key words: focal adhesion formation, pp125\textsuperscript{FAK}, tyrosine phosphorylation, vinculin, SMC

INTRODUCTION

Adhesion of cells to the extracellular matrix (ECM) is a critical step in such diverse biological processes as normal cell growth, development and differentiation, metastasis and embryological development (for review see Hynes, 1992). It is clear that many of the interactions between cells and the ECM are mediated by the integrin family of cell surface receptors. Currently, 15 \( \alpha \) and 8 \( \beta \) subunits have been identified that can associate in various heterodimers such that the specific combination determines ligand binding specificity. Most integrins are expressed on a wide variety of cell types and most cells can express a large repertoire of different integrins.

One concept of integrin-mediated signalling suggests that integrins organise the cytoskeleton into a scaffold that supports interactions between components of the intracellular signalling machinery. Integrins are concentrated in focal adhesions, structures formed at regions where the cell is in close contact with the extracellular substratum. Whilst the extracellular domains of the integrin molecules form the
ligand binding sites, the cytoplasmic domain of at least the β1 integrin has been shown to interact with two cytoskeletal elements: α-actinin and talin (Otey et al., 1990; Horwitz et al., 1986). These proteins interact with several other focal adhesion-associated proteins including actin and vinculin, thereby establishing a direct, physical link between the ECM and the cytoskeleton (Jones et al., 1989; Burridge et al., 1988; McGregor et al., 1994). However, it is becoming increasingly apparent that integrin-mediated signalling is far more complex than simply providing a link between the ECM and the cytoskeleton. Indeed, integrins have been implicated in such diverse signalling events as the control of intracellular pH and intracellular calcium concentration (Schwartz et al., 1991; Jaconi et al., 1991) and in the tyrosine phosphorylation of numerous cellular proteins (Kornberg et al., 1991; Guan and Shalloway, 1992; Huang et al., 1993).

Since integrins do not appear to possess intrinsic enzymatic activity, the increase in cellular tyrosine phosphoprotein levels associated with integrin engagement necessitates the involvement of integrin-regulatable protein tyrosine kinases. One such tyrosine kinase is pp125FAK, which localises to focal adhesions and is itself phosphorylated on tyrosine in RSV-transformed fibroblasts (Schaller et al., 1992) and in response to integrin clustering or integrin-mediated adhesion (Kornberg et al., 1991; Guan and Shalloway, 1992; Hanks et al., 1992; Schaller et al., 1992; Burridge et al., 1992). The increase in pp125FAK tyrosine phosphorylation correlates with increased intrinsic tyrosine kinase activity (Lipfert et al., 1992; Schaller et al., 1992). The presence of tyrosine-phosphorylated proteins in focal adhesions and the regulation of their tyrosine phosphorylation in response to cell adhesion has led to the suggestion that pp125FAK plays a critical role in focal adhesion assembly (Burridge et al., 1992; Schaller et al., 1992).

Interactions between the ECM and vascular smooth muscle cells (SMC) appear to be involved in many key steps in the development of the atherosclerotic lesion. SMC retain the ability to shift from a contractile to a synthetic phenotype, the switch occurring most dramatically during atherogenesis and when SMC are established in culture (for review see Ross, 1993). ECM components, such as fibronectin and laminin appear to mediate SMC adhesion and to promote cell migration and proliferation, critical events involved in the development of the disease (Ross, 1986; Schwartz et al., 1986). Indeed, adherence to certain ECM molecules promotes endogenous fibronectin expression, which is capable of supporting SMC proliferation and therefore accelerated plaque formation (Hedin et al., 1988). In an attempt to elucidate the mechanisms of ECM signalling that dictate SMC behaviour during atherogenesis, we have investigated the activation of pp125FAK in cultures of mouse aortic smooth muscle cells (MASMC). In this report, we present novel data concerning pp125FAK tyrosine kinase activation in MASMC in response to overnight culture and demonstrate that this treatment promoted the assembly of F-actin stress fibres and focal adhesions. We have shown that adhesion of MASMC to fibronectin induced F-actin stress fibre and focal adhesion formation within 30 minutes. However, adhesion of MASMC to fibronectin, although capable of supporting focal adhesion formation, failed to promote pp125FAK tyrosine kinase activity. MASMC exposed to MASMC-elaborated ECM for the same time period possess a poorly defined F-actin stress fibre network and rudimentary focal adhesions. However, pp125FAK tyrosine kinase was highly active in these cells. Thus we have demonstrated the existence of two adhesion-mediated situations in MASMC; one in which fibronectin promotes cytoskeletal reorganisation in the absence of associated pp125FAK tyrosine kinase activity and the other in which cells adhering to MASMC-ECM display elevated pp125FAK tyrosine kinase activity in association with an impaired ability to promote F-actin stress fibre and focal adhesion formation. These observations suggest that in MASMC, pp125FAK activity is not required for ECM-induced F-actin stress fibre and focal adhesion formation.

**MATERIALS AND METHODS**

**Antibodies**

The recombinant PY20 anti-phosphotyrosine monoclonal antibody, RC20 (Transduction Labs., distributed through Affiniti, Nottingham, UK), was used at 1:2500 for western blot analysis. For immunofluorescence the monoclonal anti-phosphotyrosine antibody, 4G10 (UBI, distributed through TCS, Buckingham, UK), and the monoclonal anti-human vinculin antibody (Sigma, Poole, Dorset, UK) were utilised at 1:50, and monoclonal anti-pp125FAK, 2A7 (UBI), at 1:100. FITC-conjugated goat anti-mouse IgG (Sigma) was used at 1:50 dilution and rhodamine-conjugated phallicidin (Dako Ltd, High Wycombe, Bucks, UK) at 1:200. For immunoprecipitations, a rabbit anti-mouse IgG (Sigma) was used at 10-fold excess.

**Cell culture**

Mouse aortic smooth muscle cells (MASMC) were isolated from aortic explants from C57BL/6J mice (Chamley-Campbell et al., 1979). MASMC and NIH 3T3 cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) (Gibco, Paisley, Scotland) supplemented with 10% foetal calf serum (FCS) (Gibco, Paisley, Scotland) supplemented with 10% foetal calf serum (FCS) (Gibco) and 1% glutamine, penicillin and streptomycin (Gibco). MASMC were routinely passaged by ECGS (Sigma, Poole, Dorset, UK), used at 1:200. For immunofluorescence studies, the cells were plated on glass coverslips or on glass eight-well multichamber slides at a plating density of 1×10⁶ cells per well for overnight incubation and at 2×10⁵ cells per well for shorter incubations. For immunoprecipitation experiments, the cells were plated to 9 cm dishes and cultured 10⁶ cells/dish for overnight incubation, or 4×10⁵ cells/dish for shorter incubations. For adhesion experiments, the cells were trypsinised, washed once in medium containing 10% FCS and resuspended in medium containing 10% FCS. For culturing under serum-free conditions, the cells were washed further in serum-free medium, resuspended and plated in serum-free medium. Coverslips or culture dishes were coated with 0.05 mg/ml bovine plasma fibronectin (Sigma) by incubation overnight at 4°C. The coverslips or dishes were washed twice in PBS, blocked in 10 mg/ml BSA (Sigma) at 37°C for a further 60 minutes and then washed twice in PBS.

**Preparation of SMC-elaborated ECM**

A total of 2×10⁶ cells were plated to 9 cm dishes and cultured overnight in the presence of 10% FCS. The MASMC-ECM-coated...
dishes were prepared according to either of two different protocols. In the first method, the cells were washed twice in Ca²⁺- and Mg²⁺-free PBS, then the cells were removed by incubation at 4°C in 0.5% EDTA, pH 8.0, and the ECM was washed several times with DMEM. The second method was performed according to Scott-Burden et al. (1989), where the cells were washed twice in PBS and once in water. The cells were then removed by brief treatment with 25 mM NH₄OH and the ECM was washed twice with PBS and twice with DMEM.

Fluorescence microscopy

For immunofluorescence microscopy, cells were washed once in PBS and fixed in PBS containing 3.7% formaldehyde for 10 minutes. The cells were then permeabilised in PBS containing 0.5% Triton X-100 for 5 minutes, blocked in PBS containing filtered FCS, to a final concentration of 10%, for 20 minutes and then rinsed in PBS. The coverslips or slides were then incubated for 60 minutes at room temperature in the appropriate dilution of primary antibody, washed extensively in PBS and incubated with a 1:50 dilution of FITC-conjugated goat anti-mouse IgG (Sigma) for a further 60 minutes. F-actin was visualised directly utilising a 1:200 dilution of rhodamine-conjugated goat anti-mouse IgG (Sigma). Coverslips or slides were washed as before and mounted using Citifluor (Citifluor Ltd, London, UK). Fluorescence was visualised using a Zeiss Axioskop microscope equipped with epifluorescence and photographed using Kodak Ektachrome 400 ASA film.

Immunoprecipitation

Cells from 9 cm dishes were rinsed in PBS, scraped into PBS and resuspended in 1 ml of lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP40, 50 mM NaF, 100 µM Na₃VO₄). To immunoprecipitate pp125FAK, the lysates were precleared with 30 µl of 50% solution of Protein G-Sepharose (Sigma) for 30 minutes. The Sepharose was then removed by centrifugation and the supernatants incubated with the anti-pp125FAK antibody for a minimum of 60 minutes at 4°C. Rabbit anti-mouse IgG (Sigma) bound to Protein G-Sepharose was then added to the samples for a further 60 minutes and the immunoprecipitates were washed extensively in lysis buffer. The immunoprecipitates were then utilised for kinase assays or the protein released for SDS-PAGE by boiling in SDS-sample buffer (0.1 M Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol and bromophenol blue) for 5 minutes.

In vitro kinase assays

Anti-pp125FAK immunoprecipitates were washed twice in kinase buffer (150 mM NaCl, 20 mM Tris-HCl, pH 7.6, 5 mM MgCl₂, 5 mM MnCl₂, 100 µM Na₃VO₄). The immunoprecipitates were then incubated in 50 µl kinase buffer containing 5 µCi [γ-³²P]ATP (Amersham, UK) per sample, for 15 minutes at room temperature. The immunoprecipitates were washed extensively in kinase buffer and then boiled for 5 minutes in SDS-sample buffer.

SDS-PAGE and western blot analysis

Immunoprecipitates were resolved in 7.5% SDS-PAGE gels. For kinase assays, the gels were stained in Coomassie blue, destained, dried and exposed to Hyperfilm X-ray film (Amersham), with or without intensifying at −70°C, as appropriate. For western blot analysis, the samples were electroblotted to nitrocellulose (Schleicher and Schuell). The filters were rinsed in wash buffer (100 mM NaCl, 10 mM Tris-HCl, pH 8, 0.1% Tween 20) and then blocked in wash buffer containing 1% BSA for 60 minutes at room temperature. The filters were then washed extensively in several changes of wash buffer and incubated with peroxidase-conjugated anti-phosphotyrosine antibody RC20 for 60 minutes. The filters were washed extensively as before and the protein was detected using a ECL chemiluminescence kit (Amersham).

RESULTS

MASMC exhibit pp125FAK tyrosine kinase activity in overnight culture

In order to investigate pp125FAK tyrosine kinase activity in MASMC under normal culture conditions, cells were cultured for 16 hours in medium containing 10% FCS. The cells were lysed, pp125FAK immunoprecipitated and an in vitro kinase assay was performed to determine pp125FAK tyrosine kinase activity, as measured by autophosphorylation (Lipfert et al., 1992; Schaller et al., 1992) (Fig. 1). A phosphorylated protein of approximately 125 kDa was detected in the anti-pp125FAK immunoprecipitates from NIH 3T3 and MASMC cell lysates (Fig. 1, lanes 4, 6, 8 and 10). This tyrosine-phosphorylated protein was not detected when the immunoprecipitations were performed in the absence of the anti-pp125FAK antibody (Fig. 1, lanes 5 and 9). Therefore, MASMC in overnight culture display an active pp125FAK tyrosine kinase. pp125FAK activity was similarly detected in cultures of human umbilical artery smooth muscle cells (HUASMC) (Fig. 1, lane 1). Comparable activation of the pp125FAK tyrosine kinase has been reported for Balb/c 3T3 cells (Hanks et al., 1992). These experiments demonstrate that MASMC in routine culture possess pp125FAK activity that can be readily detected.

Overnight cultures of MASMC exhibit F-actin stress fibres and focal adhesions

To determine whether overnight cultures of MASMC possess focal adhesions, MASMC were cultured for 16 hours in the presence of 10% FCS on glass coverslips or multichamber slides. Cells were double-stained for F-actin and vinculin (Fig. 2A and B), F-actin and pp125FAK (Fig. 2C and D), and F-actin and pp125FAK (lane 1), without anti-pp125FAK (lane 5); and MASMC immunoprecipitated with the anti-pp125FAK antibody. An in vitro kinase assay was similarly detected in cultures of human umbilical artery smooth muscle cells (HUASMC) (Fig. 1, lane 1). Comparable activation of the pp125FAK tyrosine kinase has been reported for Balb/c 3T3 cells (Hanks et al., 1992). These experiments demonstrate that MASMC in routine culture possess pp125FAK activity that can be readily detected.

![Fig. 1. pp125FAK tyrosine kinase activity in overnight cultures of MASMC, HUASMC and NIH 3T3 cells. MASMC and NIH 3T3 cells were cultured in medium containing 10% FCS, and HUASMC in medium containing 16% FCS, for 16 hours. The cells were lysed and immunoprecipitated with the anti-pp125FAK antibody. An in vitro kinase assay was performed on the immunoprecipitates and the samples were analysed by 7.5% SDS-PAGE and autoradiography. HUASMC immunoprecipitated with anti-pp125FAK (lane 1), without anti-pp125FAK (lane 2); NIH 3T3 cells immunoprecipitated with anti-pp125FAK without preclearance (lane 3), with preclearance (lanes 4 and 6), without anti-pp125FAK (lane 5); and MASMC immunoprecipitated with anti-pp125FAK without preclearance (lane 7), with preclearance (lanes 8 and 10) and without anti-pp125FAK (lane 9). Molecular mass markers (kDa) are shown on the right.](image-url)
and phosphotyrosine (Fig. 2E and F). When cultured under these conditions, MASMC displayed prominent F-actin stress fibres (Fig. 2A, C, and E). Furthermore, immunostaining with the anti-vinculin antibody revealed the presence of large, well-formed focal adhesions (Fig. 2B). As has been reported for other adherent cell types, pp125FAK exhibited a characteristic focal adhesion distribution in MASMC (Fig. 2D). Immunostaining with the anti-phosphotyrosine antibody revealed that phosphotyrosine-containing proteins were concentrated in focal adhesions (Fig. 2F). These data are consistent with previous reports of focal adhesion-associated tyrosine phosphorylation (Maher et al., 1985; Burridge et al., 1992; Hanks et al., 1992).

**Adherence of MASMC to fibronectin promotes stress fibre and focal adhesion formation**

In order to investigate the assembly of the actin stress fibre network and focal adhesions in response to fibronectin, MASMC were plated onto fibronectin-coated coverslips or chamber-slides in the absence of serum for 30 and 60 minutes. At early time points following plating (15 minutes), the cells

![Fig. 2. MASMC exhibit F-actin stress fibre and focal adhesion formation in response to overnight culture. MASMC cultured in medium containing 10% FCS for 16 hours were fixed and double-stained for F-actin and vinculin (A and B), F-actin and pp125FAK (C and D), and F-actin and phosphotyrosine (E and F). Bar, 10 μm.](image-url)
were strongly adhered to the substratum, but were still well-rounded and no focal adhesions were detected (data not shown). By 30 minutes the cells were displaying early signs of spreading, including small lamellipodia and membrane protrusions. The cells were fixed and double-stained for F-actin and vinculin (Fig. 3A-D), F-actin and pp125FAK (Fig. 3E-H), and phosphotyrosine, 30 minutes (J and K) and 60 minutes (L and M). Bar, 10 μm.

Fig. 3. Adherence of MASMC to fibronectin promotes stress fibre and focal adhesion formation. MASMC were plated onto coverslips coated with 0.05 mg/ml fibronectin, in the absence of serum for 30 and 60 minutes. The cells were fixed and double-stained for: F-actin and vinculin, 30 minutes (A and B), and 60 minutes (C and D); F-actin and pp125FAK, 30 minutes (E and F), and 60 minutes (G and H); and F-actin and phosphotyrosine, 30 minutes (J and K) and 60 minutes (L and M). Bar, 10 μm.
Adherence of MASMC to fibronectin fails to activate pp125\(^{Fak}\) tyrosine kinase

To determine whether pp125\(^{Fak}\) was activated in MASMC in response to adhesion to fibronectin, MASMC were plated onto fibronectin-coated dishes in the presence of 10% FCS and the cells were lysed 15, 30 and 60 minutes following plating. The lysates were immunoprecipitated with the anti-pp125\(^{Fak}\) antibody and the immunoprecipitates were separated by 7.5% SDS-PAGE and analysed by western blotting with the anti-pp125\(^{Fak}\) antibody. Adherence to fibronectin for 15, 30 and 60 minutes (lanes 1-3) or 16 hours of culture on tissue culture plastic (lane 4). Molecular mass markers (kDa) are shown on the left.

The immunoprecipitates were separated by 7.5% SDS-PAGE and analysed by western blotting with the anti-phosphotyrosine antibody and the immunoprecipitates analysed by SDS-PAGE and anti-phosphotyrosine western blotting (Fig. 4). Immunoprecipitated pp125\(^{Fak}\) was not detected by the anti-phosphotyrosine antibody in lysates from cells cultured on fibronectin for between 15 and 60 minutes. Indeed, pp125\(^{Fak}\) was only phosphorylated on tyrosine in response to overnight culture on plastic (Fig. 4, lane 4), as was seen in Fig. 1. The kinetics of pp125\(^{Fak}\) tyrosine phosphorylation following exposure of quiescent Swiss 3T3 cells to neuropeptides, such as bombesin, are extremely rapid (Sinnett-Smith et al., 1993), with an increase in pp125\(^{Fak}\) tyrosine phosphorylation apparent as early as 15 seconds following treatment. It is extremely unlikely that pp125\(^{Fak}\) activation in MASMC, in response to adhesion, follows similar kinetics, since the majority of the cells become adherent only after exposure to fibronectin for between 10 and 15 minutes.

In order to determine whether the kinetics of adhesion-induced pp125\(^{Fak}\) tyrosine phosphorylation are somewhat delayed in MASMC, parallel cultures of MASMC were plated onto fibronectin, in the absence of serum and harvested at hourly intervals between 2 and 6 hours. In vitro kinase assays were performed on pp125\(^{Fak}\) immunoprecipitates and the reactions were analysed by SDS-PAGE (Fig. 5). The results in Fig. 5 indicate that even prolonged exposure of MASMC to fibronectin does not lead to pp125\(^{Fak}\) activation. The variably tyrosine-phosphorylated protein of approximately 90 kDa has been observed previously and most likely represents a single dimer between the heavy and light Ig chains of the antibody (Lipfert et al., 1992). In order to explore the relationship between adherence and pp125\(^{Fak}\) activation more fully, the early time course of adherence to fibronectin was repeated and additional samples were prepared following overnight adherence to fibronectin. As a positive control, HUVEC were plated on fibronectin for 60 minutes, conditions under which pp125\(^{Fak}\) is activated in this cell type (Romer et al., 1994). pp125\(^{Fak}\) was immunoprecipitated, an in vitro kinase assay performed and the reactions were analysed by SDS-PAGE (Fig. 6). As before, adherence of MASMC to fibronectin, even when extended overnight, was not sufficient to promote pp125\(^{Fak}\) tyrosine kinase activation (Fig. 6B). Although the level of pp125\(^{Fak}\) autophosphorylation associated with the 30 minute time point appears to be slightly stronger than that associated with the 15 and 60 minute time points (Fig. 6, lanes 5-7), this merely reflects low level fluctuation in the basal activity.
of pp125FAK. We were able to confirm that adherence of HUVEC to fibronectin caused pp125FAK autophosphorylation (Fig 6A), indicating that the failure of fibronectin to activate pp125FAK in MASMC was not due to inappropriate experimental conditions. Thus, under conditions shown to promote F-actin stress fibre and focal adhesion formation in MASMC, pp125FAK in these cells remains inactive.

**Adherence of MASMC to MASMC-elaborated ECM strongly activates pp125FAK tyrosine kinase**

Overnight adherence to fibronectin reduced the pp125FAK activity associated with routine overnight culture (Fig 6B, lanes 2-4). Therefore during overnight culture, MASMC are capable of establishing a situation that promotes pp125FAK activation that is apparently negatively regulated by exogenous fibronectin. It is conceivable that MASMC deposit their own combination of ECM molecules that are responsible for activating pp125FAK. Alternatively, MASMC may secrete factor(s) into their conditioned medium, such as growth factors or neuroptides, which are known to activate pp125FAK in quiescent Swiss 3T3 cells (Rankin and Rozengurt, 1994; Zachary et al., 1992; Sinnett-Smith et al., 1993). To investigate these possibilities, MASMC-elaborated ECM-coated dishes (MASMC-ECM) were prepared and conditioned medium was harvested (see Materials and Methods). Freshly trypsinised MASMC were plated on MASMC-ECM dishes in the presence of 10% FCS for 30, 45 and 60 minutes (Fig. 7, lanes 6-8). Alternatively, cells were plated onto tissue culture plastic (Fig. 7 lanes 3 and 4) or on fibronectin-coated dishes (Fig. 7, lane 5) in the presence of MASMC-conditioned medium. The results in Fig. 7 indicate that medium conditioned by overnight cultures of MASMC is not able to promote pp125FAK activity alone or in combination with fibronectin. In contrast, adherence of MASMC to MASMC-elaborated ECM results in pp125FAK activation, activation occurring within the same time course in which fibronectin-mediated adhesion was non-stimulatory. These results indicate that the original time course chosen to study the effects of adhesion to fibronectin, as shown in Figs 4 and 6, was appropriate. The increase in phosphotyrosine content of pp125FAK associated with short-term exposure to MASMC-ECM represents pp125FAK tyrosine kinase activation rather than merely reflecting an elevation in pp125FAK protein content. Therefore, in MASMC, pp125FAK becomes activated in response to adhesion to MASMC-ECM as a direct consequence of the action of the ECM molecules themselves and/or factors which are associated tightly with the ECM.

**Activation of pp125FAK tyrosine kinase by MASMC-ECM does not require serum**

To determine whether the MASMC-ECM signal acts coordinately with factor(s) in serum in order to achieve pp125FAK activation, freshly trypsinised cells were plated to MASMC-ECM for between 10 and 60 minutes, in the presence or

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**Fig. 6.** Adherence to fibronectin promotes the activation of pp125FAK tyrosine kinase in HUVEC, without activating pp125FAK tyrosine kinase in MASMC. Freshly trypsinised MASMC were plated on 9 cm dishes coated with 0.05 mg/ml fibronectin for 15, 30, 60 minutes and 16 hours in the presence of 10% FCS, for 16 hours in the absence of serum, or cultured on tissue culture plastic in 10% FCS for 16 hours. Freshly trypsinised HUVEC were plated on fibronectin-coated dishes or retained in suspension for 60 minutes. The cells were lysed and the lysates immunoprecipitated with the anti-pp125FAK antibody. An in vitro kinase assay was performed on the immunoprecipitates and the reactions analysed by 7.5% SDS-PAGE and autoradiography. (A) Anti-pp125FAK immunoprecipitates from HUVEC retained in suspension (lane 1) or following adherence to fibronectin for 60 minutes (lane 2). (B) Anti-pp125FAK immunoprecipitates from MASMC cultured on tissue culture plastic in 10% FCS for 16 hours; no anti-pp125FAK (lane 1); with anti-pp125FAK (lane 2); MASMC cultured for 16 hours on fibronectin in the absence (lane 3) or presence (lane 4) of 10% FCS; and MASMC cultured on fibronectin for 15, 30 and 60 minutes in the presence of 10% FCS (lanes 5-7). Molecular mass markers (kDa) are shown on the left.

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**Fig. 7.** Adherence of MASMC to MASMC-elaborated ECM activates pp125FAK tyrosine kinase. MASMC-elaborated ECM-coated plates were prepared from MASMC cultured for 16 hours in the presence of 10% FCS (as described in Materials and Methods) and the conditioned medium was harvested. freshly trypsinised MASMC were plated on 9 cm dishes coated with 0.05 mg/ml fibronectin in the presence of 10% FCS or MASMC-conditioned medium. The results in Fig. 7 indicate that the original time course chosen to study the effects of adhesion to fibronectin, as shown in Figs 4 and 6, was appropriate. The increase in phosphotyrosine content of pp125FAK associated with short-term exposure to MASMC-ECM represents pp125FAK tyrosine kinase activation rather than merely reflecting an elevation in pp125FAK protein content. Therefore, in MASMC, pp125FAK becomes activated in response to adhesion to MASMC-ECM as a direct consequence of the action of the ECM molecules themselves and/or factors which are associated tightly with the ECM.
with the anti-pp125FAK antibody. An in vitro kinase assay was performed on the immunoprecipitates and the reactions analysed by 7.5% SDS-PAGE followed by autoradiography. Anti-pp125FAK immunoprecipitates from MASMC cultured for 16 hours in 10% FCS; with anti-pp125FAK (lane 1); without anti-pp125FAK (lane 2), following adherence to fibronectin for 60 minutes in 10% FCS (lane 3); following adherence to MASMC-ECM in 10% FCS for 10 minutes (lane 4); 30 minutes (lane 5) and 60 minutes (lane 6); and following adherence to MASMC-ECM in 0.1% FCS for 30 minutes (lane 7) and 60 minutes (lane 8). Molecular mass markers (kDa) are shown on the left.

absence of 10% FCS. The pp125FAK was immunoprecipitated and an in vitro assay was performed. The results in Fig. 8 indicate that MASMC-elaborated ECM alone is sufficient to promote the activation of pp125FAK (Fig. 8, compare lanes 4-6 with 7 and 8). Furthermore, pp125FAK activity was maximal as early as 10 minutes (Fig. 8, lane 4) coincident with cell adhesion and prior to the onset of cell spreading.

**MASMC do not form F-actin stress fibres and focal adhesions under conditions which promote pp125FAK tyrosine kinase activation**

To determine whether MASMC produce F-actin stress fibres and focal adhesions in response to pp125FAK proactivating conditions, MASMC were plated on MASMC-ECM-coated multichamber slides in the absence of 10% FCS for 30 and 60 minutes. Cells were double-stained for F-actin and vinculin (Fig. 9A-D), F-actin and pp125FAK (Fig. 9E-H), and F-actin and phosphotyrosine (Fig. 9I-M). The process of cells spreading on MASMC-ECM is significantly slower than on fibronectin (compare Fig. 9 with Fig. 3), although at later times, the cells on MASMC-ECM become well spread and morphologically indistinguishable from cells plated onto fibronectin (data not shown). At 30 minutes, vinculin expression is associated with peripheral, newly forming focal adhesions (Fig. 9B) and pp125FAK appears to be concentrated in peripheral patches associated with primary, rudimentary sites of adhesion (Fig. 9F). Antiphosphotyrosine staining is associated with large, cytoplasmic podosomal-like structures (Fig. 9K). F-actin is either localised to peripheral patches (Fig. 9E) or arranged concentrically around the cell periphery (Fig. 9J), although some weakly staining F-actin stress fibres can be observed (Fig. 9A). By 60 minutes, F-actin stress fibres are slightly more pronounced (Fig. 9G and L) and vinculin and pp125FAK expression is associated with peripheral, newly formed focal adhesions (Fig. 9D,H), with anti-phosphotyrosine staining still largely associated with the podosomal-like structures (Fig. 9M). This variable immunofluorescence localisation observed for F-actin, vinculin, pp125FAK and phosphotyrosine reflects the degree of flattening and elongation of cells involved in the early stages of the spreading process. Thus, MASMC exposed to MASMC-elaborated ECM, conditions shown to promote pp125FAK tyrosine phosphorylation, display a poorly defined F-actin stress fibre network and only rudimentary focal adhesions. Similar results were obtained when cells were plated on MASMC-ECM in the presence of 10% FCS (data not shown). These results support our original proposal that pp125FAK tyrosine kinase activity does not appear to correlate with F-actin stress fibre assembly and focal adhesion formation.

**DISCUSSION**

We have demonstrated that the signal(s) resulting from integrin engagement following adherence of MASMC to fibronectin is sufficient to promote F-actin stress fibre and focal adhesion formation. Furthermore, we have detected the recruitment of pp125FAK and phosphotyrosine-containing proteins into the newly formed adhesions. These data are in agreement with several previously published reports, including that of Burridge et al. (1992), who studied the increase in tyrosine phosphorylation associated with the adhesion of rat embryo fibroblasts (REF) and mouse 3T3 cells to fibronectin. Furthermore, these workers demonstrated that treatment with the tyrosine kinase inhibitor Herbimycin A interfered with the increase in tyrosine phosphorylation associated with adhesion and inhibited the formation of F-actin stress fibres and focal adhesions. The suggestion that tyrosine phosphorylation is directly involved in the formation of stress fibres and focal adhesions was further corroborated by the findings of Barry and Critchley (1994), who showed that treatment of quiescent Swiss 3T3 cells lacking F-actin stress fibres and focal adhesions (Ridley and Hall, 1992) with the tyrosine phosphatase inhibitor, vanadyl hydroperoxide, induces the dramatic reappearance of the stress fibre assembly.

These reports and others present evidence that one of the proteins which is tyrosine phosphorylated in response to adhesion is pp125FAK. It was proposed that pp125FAK is responsible for the increased cellular tyrosine phosphorylation detected in focal adhesions following adherence and therefore plays a crucial role in this cytoskeletal re-organisation. However, our data suggest that in MASMC, the increase in focal adhesion-association of phosphotyrosine-containing proteins in response to fibronectin-mediated adhesion is not a consequence of pp125FAK activation, indicating that a distinct tyrosine kinase(s) is responsible. The ability of tyrosine kinase inhibitors to interfere with focal adhesion formation in REF (Burridge et al., 1992) could also be interpreted as the inhibition of an upstream tyrosine kinase. In support of a role for another tyrosine kinase in focal adhesion formation, Romer et al. (1994) investigated the effects of two tyrosine kinase...
inhibitors displaying different patterns of specificity; tyrphostins, which inhibit all tyrosine kinases, and Herbimycin A, which acts specifically upon pp60src family kinases. When the inhibitors were used on isolated pp125FAK immunoprecipitates, they produced demonstrably different effects on its kinase activity, with tyrphostins completely abrogating autophosphorylation and Herbimycin A causing only a slight inhibition. However, pretreatment of whole cells with the inhibitors resulted in more comparable inhibition. These results could be interpreted to indicate that within cells, Herbimycin A inhibits a pp60src family kinase, which phosphorylates and activates pp125FAK. Indeed, it has been demonstrated that pp125FAK acts as a substrate for pp60src in RSV-transformed cells (Kanner et al., 1990) and that pp125FAK forms a stable association with pp59fyn in normal CE cells (Cobb et al., 1994). Furthermore, in platelets, binding of fibrinogen to the αIIbβ3 receptor initiates tyrosine phosphorylation of a number of proteins, which precedes the tyrosine phosphorylation of

Fig. 9. Adherence of MASMC to MASMC-ECM results in an impaired ability to promote F-actin stress fibre assembly and focal adhesion formation. MASMC were plated onto MASMC-ECM-coated multichamber slides, in the absence of serum for 30 and 60 minutes. The cells were fixed and double-stained for: F-actin and vinculin, 30 minutes (A and B) and 60 minutes (C and D); F-actin and pp125FAK, 30 minutes (E and F) and 60 minutes (G and H); and F-actin and phosphotyrosine, 30 minutes (J and K) and 60 minutes (L and M). Bar, 10 μm.
pp125FAK, which is further dependent upon platelet aggregation (Huang et al., 1993). These results could be interpreted to suggest the involvement of an additional kinase, which might in turn regulate pp125FAK activity.

Mutational analysis of pp125FAK has been performed to investigate the relationship between pp125FAK activity and cell morphology (Hildebrand et al., 1993). Since both p41FRNK, the C-terminal non-catalytic domain of pp125FAK produced by alternative splicing (Schaller et al., 1993), and a kinase-inactive mutant of pp125FAK localise correctly to focal adhesions, pp125FAK autophosphorylation appears not to play a role in pp125FAK targeting. Furthermore, the overexpression of p41FRNK or the kinase-inactive mutant does not appear to affect normal cell morphology or growth (Schaller et al., 1993); this apparent lack of phenotype suggests that pp125FAK kinase activity is not a prerequisite for focal adhesion formation.

It is conceivable that the signalling pathways responsible for mediating cytoskeletal re-organisation in response to adhesion are in fact cell type-specific, and that in MASMC focal adhesion formation occurs independently of tyrosine phosphorylation. Indeed, components of the focal adhesion may become tyrosine phosphorylated as a direct consequence of, rather than contributing to, cytoskeletal re-organisation. In REF, Burridge et al. (1992) indicated that tyrosine phosphorylation is not sufficient for focal adhesions to develop and that at least in primary fibroblasts, PKC-mediated phosphorylation changes may be an important signalling mechanism in the assembly of focal adhesions and stress fibres (Woods and Couchman, 1992). The localisation of PKCδ to newly formed focal adhesions in Swiss 3T3 cells by Barry and Critchley (1994) supports this potential role for PKC and therefore serine/threonine phosphorylation in focal adhesion assembly and cell spreading. Schaller et al. (1993) have shown that p41FRNK is phosphorylated on serine/threonine in an ECM-dependent manner and suggested that pp125FAK may be phosphorylated in a similar fashion. Furthermore, they revealed that pp125FAK and p41FRNK contain a consensus phosphorylation site for PKC. Therefore, the serine/threonine phosphorylation of residues in the C terminus of pp125FAK may promote the recruitment of other cellular proteins to the focal adhesions, thereby acting as a docking protein. Indeed, pp125FAK could perform this function independently of its tyrosine kinase activity.

A role for pp125FAK activation in cell migration was suggested by the observation of increased tyrosine phosphorylation in focal adhesions associated with the migration of HUVEC into experimental wounds (Romer et al., 1994). Recent evidence demonstrated that hepatocyte growth factor induces tyrosine phosphorylation of pp125FAK and promotes migration by oral squamous cell carcinomas (Matsumoto et al., 1994). However, cell migration is normally characterised by the absence of focal adhesions, suggesting that rather than playing a role in the assembly of focal adhesions, pp125FAK may actually initiate the disassembly of focal adhesions, a process required prior to cell mobilisation and locomotion.

We have also demonstrated that overnight cultures of MASMC possess active pp125FAK. This observation would be consistent with the proposal that during overnight culture, MASMC establish a situation which promotes pp125FAK activity. In support of this proposal, we have shown that adhesion of MASMC to MASMC-elaborated ECM leads to the activation of pp125FAK tyrosine kinase. Furthermore, MASMC exposed to MASMC-ECM in short-term culture display a poorly defined F-actin stress fibre network and rudimentary focal adhesions. Interestingly, elevated pp125FAK tyrosine kinase activity in the presence of reduced numbers of focal adhesions and F-actin stress fibres has similarly been observed following Rous sarcoma virus transformation of fibroblasts (Guan and Shalloway, 1992). Thus we have demonstrated the existence of two adhesion-mediated situations in MASMC; one in which fibronectin promotes cytoskeletal reorganisation in the absence of pp125FAK tyrosine kinase activity and the other in which cells adhering to MASMC-ECM display elevated pp125FAK tyrosine kinase activity in association with an impaired ability to promote F-actin stress fibre and focal adhesion formation.

SMC in culture have the capacity to synthesise a complex array of ECM proteins in response to different stimuli (Hedin et al., 1988). Therefore in MASMC, pp125FAK tyrosine kinase may be activated not by engagement of a single integrin species, but through the coordinate engagement of a combination of different integrins activated through the autocrine expression of a heterogeneous population of ECM molecules. Alternatively, MASMC may respond in an non-integrin-mediated fashion to activator(s) which are tightly associated with the ECM. The activation of pp125FAK by treatment of quiescent Swiss 3T3 cells with growth factors, such as PDGF and neuropeptides, is consistent with the existence of non-integrin-mediated pathways for pp125FAK activation (Rankin and Rozengurt, 1994; Zachary et al., 1992; Sinnett-Smith et al., 1993). It has been demonstrated that rat aortic SMC synthesise and deposit heparin-binding basic FGF (bFGF) in their ECM (Speir et al., 1991). Preliminary evidence utilising anti-bFGF antibodies indicates that ECM-associated bFGF is not responsible for MASMC-ECM mediated pp125FAK tyrosine kinase activation (data not shown). It has recently become apparent that activation of pp125FAK in platelets in fact requires coordinate signalling through integrin and agonist receptors (Shattil et al., 1994). Identification of the additional signalling mechanisms required to promote pp125FAK tyrosine kinase activation in MASMC is in progress.

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