The Assembly of Integrin Adhesion Complexes Requires Both Extracellular Matrix and Intracellular rho/rac GTPases

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Abstract. Interaction of cells with extracellular matrix via integrin adhesion receptors plays an important role in a wide range of cellular functions, for example cell growth, movement, and differentiation. Upon interaction with substrate, integrins cluster and associate with a variety of cytoplasmic proteins to form focal complexes and with the actin cytoskeleton. Although the intracellular signals induced by integrins are at present undefined, it is thought that they are mediated by proteins recruited to the focal complexes. It has been suggested, for example, that after recruitment to focal adhesions $p125^{FAK}$ can activate the ERK1/2 MAP kinase cascade. We have previously reported that members of the rho family of small GTPases can trigger the assembly of focal complexes when activated in cells. Using microinjection techniques, we have now examined the role of the extracellular matrix and of the two GTP-binding proteins, rac and rho, in the assembly of integrin complexes in both mouse and human fibroblasts. We find that the interaction of integrins with extracellular matrix alone is not sufficient to induce integrin clustering and focal complex formation. Similarly, activation of rho or rac by extracellular growth factors does not lead to focal complex formation in the absence of matrix. Focal complexes are only assembled in the presence of both matrix and functionally active members of the rho family. In agreement with this, the interaction of integrins with matrix in the absence of rho/rac activity is unable to activate the ERK1/2 kinases in Swiss 3T3 cells. In fact, ERK1/2 can be activated fully by growth factors in the absence of matrix and it seems unlikely, therefore, that the adhesion dependence of fibroblast growth is mediated through the ras/MAP kinase pathway. We conclude that extracellular matrix is not sufficient to trigger focal complex assembly and subsequent integrin-dependent signal transduction in the absence of functionally active members of the rho family of GTPases.

INTEGRINS are ubiquitous α/β heterodimeric transmembrane glycoproteins, which act as adhesion receptors involved in both cell–matrix and cell–cell interactions (Hynes, 1992). There are at least 14 different α subunits and eight different β subunits, and more than 20 different αβ combinations have been described. Interaction between integrins and extracellular matrix (ECM)[1] is known to be important in a variety of cellular processes in both adult and embryo (Hynes, 1992; Adams and Watt, 1993; Clark and Brugge, 1995). In particular, many cell types show an anchorage-dependent phenotype in culture where cell growth is dependent on both soluble mitogens and adhesion via integrins to ECM. Cell cycle progression through G1 leading to S requires integrin-dependent signals that are necessary for specific changes in gene transcription in late G1 (Folkman and Moscona, 1978; Guadagno and Assoian, 1991; Guadagno et al., 1993; Hansen et al., 1994). Exit of cells from G0 into G1 may also require integrin-ECM interactions (Schwartz et al., 1991; McNamee et al., 1993; Hansen et al., 1994). In contrast to this many transformed cells lose anchorage-dependent requirement for growth and can proliferate readily in suspension; for example, in soft agar (Shin et al., 1975).

Cellular attachment to extracellular matrix results in clustering of integrins and assembly of multi-molecular focal complexes associated with the actin cytoskeleton. These structures are believed to be involved in integrin-mediated signal transduction events (Burridge et al., 1988; Turner and Burridge, 1991; Sastry and Horwitz, 1993). The focal complexes contain a large number of cytoplasmic-derived proteins, some of which (e.g., talin, vinculin, and α-actinin) appear to be mainly structural, whilst others (e.g., $p125^{FAK}$, src, and tensin) are likely to play a more active role in interacting with the intracellular milieu. Relatively little is known about how the various components of these com-

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1. *Abbreviations used in this paper*: ECL, enhanced chemiluminescence; ECM, extracellular matrix; FN, fibronectin; GST, glutathione-*S*-transferase; LPA, lysophosphatidic acid; MAP, mitogen-activated protein; MBP, myelin basic protein; PLL, poly-L-lysine; SFM, serum-free medium; VN, vitronectin.

plexes interact in vivo, and even less about how assembly is regulated. It is apparent, however, that the nature of the interactions between the components of focal complexes is extremely complex. For example, in addition to binding to α-actinin and talin, vinculin has recently been shown to bind actin (Gilmore and Burridge, 1995; Johnson and Craig, 1995). In turn, α-actinin and talin are believed to bind both to actin and to integrin cytoplasmic tails (Horwitz et al., 1986; Otey et al., 1990).

It has been widely proposed that integrin–matrix interactions influence cell behavior by activating intracellular signal transduction pathways (Juliano and Haskill, 1993; Clark and Brugge, 1995). There is strong evidence to suggest that some of these signaling events require both an intact cytoskeleton and the assembly of focal complexes (Lipfert et al., 1992; Bockholt and Burridge, 1993; Haimovich et al., 1993; Huang et al., 1993; Seufferlein and Rozengurt, 1994). A good candidate for mediating signal transduction is the nonreceptor tyrosine kinase $p125^{FAK}$, which is recruited to focal complexes upon attachment of cells to ECM (Schaller and Parsons, 1994). The function of $p125^{FAK}$ is, however, unclear though it can act to recruit SH2-containing proteins such as src and Grb-2, the adaptor protein for the ras guanine exchange factor mSOS1 (Schlaepfer et al., 1994). A link to the ras signaling pathway is also supported by a number of reports indicating that the interaction of cells with fibronectin results in activation of the two mitogen-activated protein (MAP) kinases, ERK-1 and ERK-2 (Chen et al., 1994; Schlaepfer et al., 1994; Morino et al., 1995; Zhu and Assoian, 1995).

It has been established that members of the rho subfamily of ras-related GTP-binding proteins are required for assembly of the actin cytoskeleton and associated focal complexes (Hall, 1994). Using Swiss 3T3 fibroblasts, as a model system, it was shown that rho regulates a signal transduction pathway linking some extracellular growth factors, such as lysophosphatidic acid (LPA) and bombesin, to the assembly of "classical" focal adhesion plaques and actin stress fibers (Ridley and Hall, 1992). Rac, a second member of the GTPase family, regulates a distinct pathway linking other growth factor receptors (e.g., those for PDGF, EGF or insulin) to the polymerization of actin at the plasma membrane to produce lamellipodia and ruffles (Ridley et al., 1992). The leading edge of the rac-induced lamellipodia is also associated with focal complexes, which are much smaller than focal adhesions plaques, but appear to contain most, if not all, the same constituent proteins (Nobes and Hall, 1995). Cdc42, a third member of the family, has been shown to trigger the assembly of actin to produce filopodia extensions; these too are associated with focal complexes (Kozma et al., 1995; Nobes and Hall, 1995). It is clear, therefore, that several different kinds of focal complex exist within a cell, each of which can be independently regulated and could contribute to integrin-dependent signal transduction pathways.

We have now examined the relative roles of extracellular matrix and intracellular rho-related GTPases in focal complex assembly. Focal complexes are assembled only in the presence of both matrix and functionally active members of the rho family. Furthermore, the interaction of integrins with matrix in the absence of rho/rac activity is insufficient to activate the ERK1/2 kinases in Swiss 3T3 cells. We conclude that extracellular matrix is not sufficient to trigger focal complex assembly and integrin-dependent signal transduction, but requires in addition functionally active members of the rho family of GTPases.

Materials and Methods

Reagents

All reagents were purchased from Sigma (Poole, UK) unless stated otherwise. Human plasma fibronectin (FN) was purchased from Blood Products Ltd (Elstree, UK), human plasma vitronectin (VN) from Life Technologies (Paisley, UK) and ProNectin F from TCS Biologicals (Buckingham, UK). PDGF-BB and peptides (GRGDS and GRADSP) were purchased from Calbiochem (Nottingham, UK).

Cell Culture

Cell culture reagents were purchased from Life Technologies unless stated otherwise. Swiss 3T3 fibroblasts were cultured as described previously by seeding at high density onto tissue culture grade plastic (Nunclon) in DMEM containing 10% FCS (Sigma, Poole, UK), penicillin, and streptomycin (Nobes and Hall, 1995). Cells were allowed to reach confluence and 7–10 d after seeding cells were serum-starved for 16 h by removing medium and replacing with serum-free medium (SFM). SFM is DME containing 0.2% $NaHCO_3$. Human dermal fibroblasts, obtained from human foreskin, were cultured in DME containing 10% FCS as described previously (Hotchin et al., 1995).

Plating Experiments

For plating experiments glass coverslips were coated with: 50 μg/ml FN, overnight at 4°C; 10 μg/ml VN, 2 h at room temperature; 10 μg/ml poly-L-lysine (PLL), 1 h at room temperature. Quiescent serum-starved Swiss 3T3 cells were detached using trypsin/EDTA solution (Life Technologies) and trypsin was neutralized using 0.5 mg/ml soybean trypsin inhibitor in SFM. Cells were washed and resuspended in SFM and allowed to attach to matrix-coated coverslips.

Suspension Experiments

Serum-starved quiescent Swiss 3T3 cells were detached from culture dishes and trypsin neutralized as described above. Cells were washed once by centrifugation and resuspended in SFM. To prevent attachment cells were incubated in SFM in culture dishes coated with 0.5 mg/ml lipid-free BSA.

Protein Expression, Purification, and Microinjection

Recombinant C3 transferase, N17rac, L61rac1, L63rho, and a chimeric rac60rho were expressed as glutathione-*S*-transferase (GST) fusion proteins, purified using glutathione-agarose and cleaved using thrombin as described elsewhere (Nobes and Hall, 1995; Ridley et al., 1992). Cells were microinjected with recombinant proteins as described elsewhere (Nobes and Hall, 1995).

Adhesion Assays

Adhesion assays were performed in plastic microtitre plates (Immulon II; Dynatech Laboratories Inc., Chantilly, VA). Wells were coated overnight at 4°C with ECM proteins, rinsed in PBS, and then free binding sites were blocked with 0.5 mg/ml heat-inactivated lipid-free BSA. Quiescent, serum-starved Swiss 3T3 cells were detached and resuspended at a density of 10^5 cells/ml in SFM. 10^4 cells were added per well and allowed to attach for 30 min. Nonadherent cells were removed by washing in PBS and the number of attached cells quantitated using the hexosaminidase assay as described elsewhere (Adams and Watt, 1990). Non specific attachment to BSA alone was quantitated and subtracted from values obtained for specific adhesion. Results from triplicate wells were expressed as relative adhesion and calculated according to the following formula $[(OD405_{matrix}-OD405_{BSA})/(OD405_{input}-OD405_{BSA})]$; where $OD405_{matrix}$ represents the value obtained for adhesion to matrix, $OD405_{BSA}$ nonspecific adhesion to BSA and $OD405_{input}$ the value obtained for 10^4 cells.

Immunofluorescence

Cells were fixed in 4% paraformaldehyde and stained for the presence of vinculin or β3 integrin using a previously described method (Nobes and Hall, 1995). Vinculin was detected using a mouse monoclonal antibody (VIN11.5; Sigma) and β3 integrin using the LM609 monoclonal antibody (Cheresh and Spiro, 1987; gift of David Cheresh, Scripps Institute, La Jolla). Results were visualized on a Zeiss Axiophot microscope and photographed using T-MAX400 film (Kodak).

MAP Kinase Assays

To determine activation of the ERK-2 MAP kinase in Swiss 3T3, cells plated on different substrates and stimulated with growth factors were solubilized in lysis buffer (20 mM Tris, pH 8.0, 5 mM $MgCl_2$, 10 mM EGTA, 1% [vol/vol] Triton X-100, 0.5% [wt/vol] deoxycholic acid, 40 mM sodium pyrophosphate, 50 mM NaF, 100 μM Na_3VO_4, 20 μg/ml leupeptin, 20 μg/ml aprotinin, and 2 mM PMSF). Equal amounts of lysate were incubated for 90 min at 4°C with a rabbit polyclonal antibody to ERK-2 (122; Howe et al., 1992; gift of Dr. Chris Marshall, Institute of Cancer Research, London) coupled to protein A–Sepharose. Immunoprecipitated material was washed twice with lysis buffer minus PMSF, once with 30 mM Tris, pH 8.0, and once with kinase buffer (30 mM Tris, pH 8.0, 20 mM $MgCl_2$, and 2 mM $MnCl_2$). The final wash was aspirated completely and then 30 μl kinase reaction mix (kinase buffer containing 10 μM ATP, 250 μg/ml myelin basic protein [MBP], and 0.5 μCi $^{32}P\gamma$-ATP) was added. The kinase reaction was allowed to proceed for 30 min at 30°C and terminated by addition of 30 μl of 2× protein sample loading buffer. The reaction mix was electrophoresed on a 15% SDS-PAGE gel, fixed, dried, and then exposed to autoradiograph film. To ensure equal amounts of ERK-2 were immunoprecipitated from each sample, equal amounts of lysate were electrophoresed on a 10% SDS-PAGE gel and transferred to Immobilon polyvinyldifluoride membrane (Millipore Corp., Bedford, MA) in 10 mM cyclohexylaminopropane sulfonic acid, pH 11.0, containing 10% (vol/vol) methanol for 16 h at 0.2A. After rinsing briefly in Tris-buffered saline containing 0.05% Tween 20 (TBS/T) the filter was incubated with 1 M glycine containing 5% (vol/vol) FCS, 5% (wt/vol) skimmed milk powder and 1% (wt/vol) ovalbumin. After a further brief rinse in TBS/T, 122 antibody, diluted 1:7,500 with TBS/T was added for 60 min. The filter was washed for 60 min in TBS/T with three changes of wash buffer and horseradish peroxidase-conjugated sheep anti–rabbit IgG, diluted 1:10,000 in TBS/T, added. After a 30-min incubation the filter was washed again and immunoreactive protein detected by enhanced chemiluminescence (ECL; Amersham International, Amersham, UK).

Results

Assembly of Focal Complexes Requires Both Extracellular Matrix and Growth Factors

To examine whether extracellular matrix alone is sufficient to induce the assembly of focal complexes in Swiss 3T3 fibroblasts, quiescent, serum-starved cells were replated in serum-free conditions, onto glass coverslips coated with either PLL (10 μg/ml) or FN (50 μg/ml). After 40 min cells were fixed and stained with an anti-vinculin antibody. As shown in Fig. 1, *a* and *d*, no focal complexes were assembled under either condition.

We have previously reported that addition of LPA to quiescent cells activates endogenous rho protein and leads to the assembly of focal adhesions while addition of platelet-derived growth factor (PDGF) activates rac and leads to the formation of focal complexes at the leading edge (Ridley and Hall, 1992; Ridley et al., 1992; Nobes and Hall, 1995). To determine the role played by extracellular matrix in these activities, quiescent cells were plated on ei-

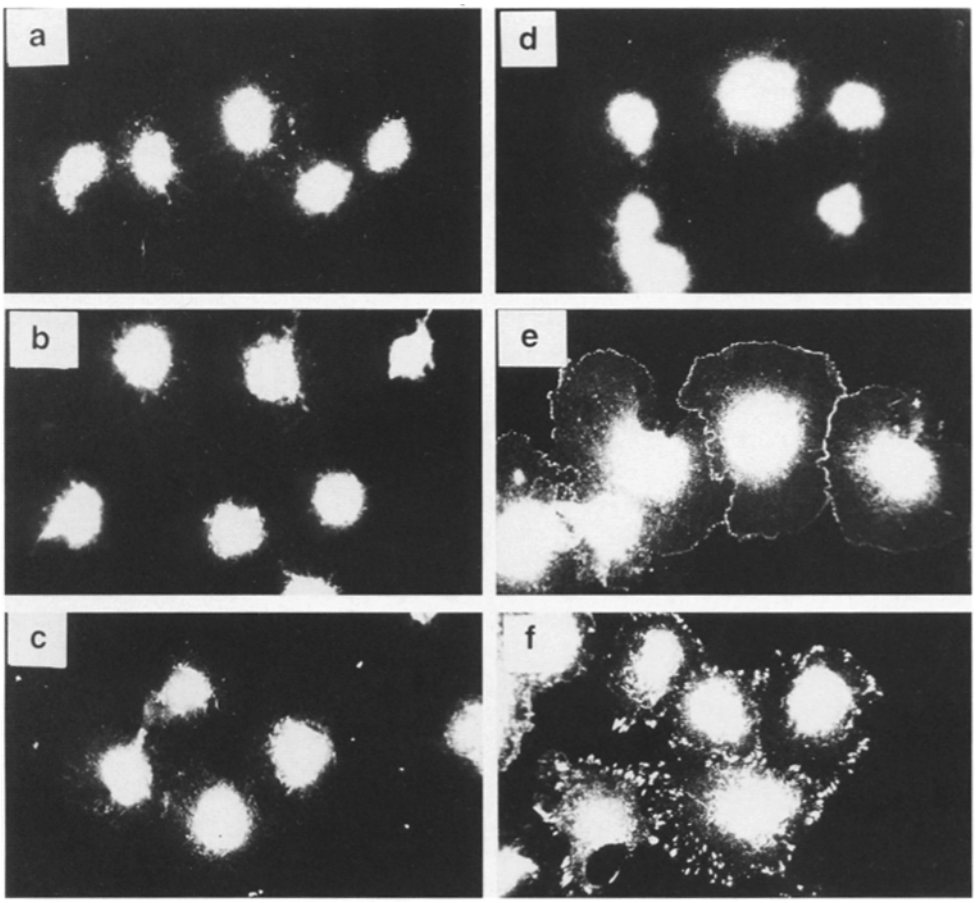

Figure 1. Focal complex formation requires interaction with ECM and growth factors. Serum-starved Swiss 3T3 cells were plated on 10 μg/ml PLL (*a–c*) or 50 μg/ml fibronectin (*d–f*) in the absence of growth factors (*a* and *d*) or in the presence of 5 ng/ml PDGF (*b* and *e*) and 100 ng/ml LPA (*c* and *f*). Cells were allowed to attach for 30 min before fixation and staining with antibody to vinculin.

ther PLL or FN and challenged with growth factors. It can be seen that PDGF (Fig. 1, *b* and *e*) and LPA (Fig. 1, *c* and *f*) only induced focal complexes when cells were plated on extracellular matrix. Microinjection of dominant negative rac protein or the rho inhibitor C3 transferase before growth factor addition, confirmed that the PDGF- and LPA-induced vinculin complexes were mediated by rac and rho, respectively, under these conditions (data not shown). The nuclear staining observed in this and other experiments (see Figs. 3 and 5) was a result of nonspecific staining by the FITC-conjugated secondary antibodies (data not shown).

Microinjection of constitutively activated recombinant rho or rac protein has been shown to induce focal complexes in the absence of growth factors (Ridley and Hall, 1992; Nobes and Hall, 1995). However, when recombinant rho or rac were injected into cells plated on PLL, no vinculin-containing complexes were induced (data not shown).

Rho-dependent Assembly of Actin Fibers Occurs in the Absence of Integrin-Matrix Interaction

In addition to looking at focal complex formation we have also used phalloidin to examine the requirement of extracellular matrix for rho dependent actin fiber assembly. Although microinjection of recombinant rho protein into cells plated on PLL did not result in focal complex assembly, it did induce the formation of actin fibers; these appeared as swirls of filaments that did not appear to be attached to the plasma membrane at discrete sites (Fig. 2 *a*). These structures are clearly differently organized from stress fibers formed on fibronectin in response to microinjection with rho (Fig. 2 *b*). Actin-rich structures were also observed around the edge of the cell, but these appeared morphologically distinct from the actin-containing membrane ruffles seen in rac-injected cells (Ridley et al., 1992). Similar results were obtained following treatment with LPA and formation of these structures could be blocked by microinjection with C3 transferase, a bacterial toxin that ADP ribosylates and inactivates rho (Ridley and Hall, 1992). We conclude that although rho-dependent formation of focal adhesion complexes is dependent on extracellular matrix, rho-dependent assembly of actin fibers is not.

Focal Complex Formation Occurs in an RGD-dependent Manner

For many cell types attachment to FN can be blocked using RGD-containing peptides, which competitively inhibit binding via $\alpha5\beta1$ or $\alpha v\beta3$ integrins (D'Souza et al., 1991). To examine whether attachment of Swiss 3T3 cells to fibronectin occurs in an RGD-dependent manner, cells were plated on the substrate for 30 min in the presence of 1 mM inhibitory (GRGDS) or noninhibitory (GRADSP) peptides. As controls, cells were also plated on PLL and ProNectin F, a synthetically derived polypeptide consisting of repeating RGD motifs. As can be seen in Fig. 3 attachment to PLL was unaffected by the inhibitory GRGDS peptide. The proportion of cells that attached under these conditions was ~60%. The efficiency of attachment to FN was similar, but this was not blocked by the inhibitory RGD-containing peptide, even at concentrations as high as 10 mM (data not shown). Attachment of cells to ProNectin F, although somewhat less efficient, was inhibited by the GRGDS, indicating that the peptide was active and capable of blocking integrin-substrate interactions.

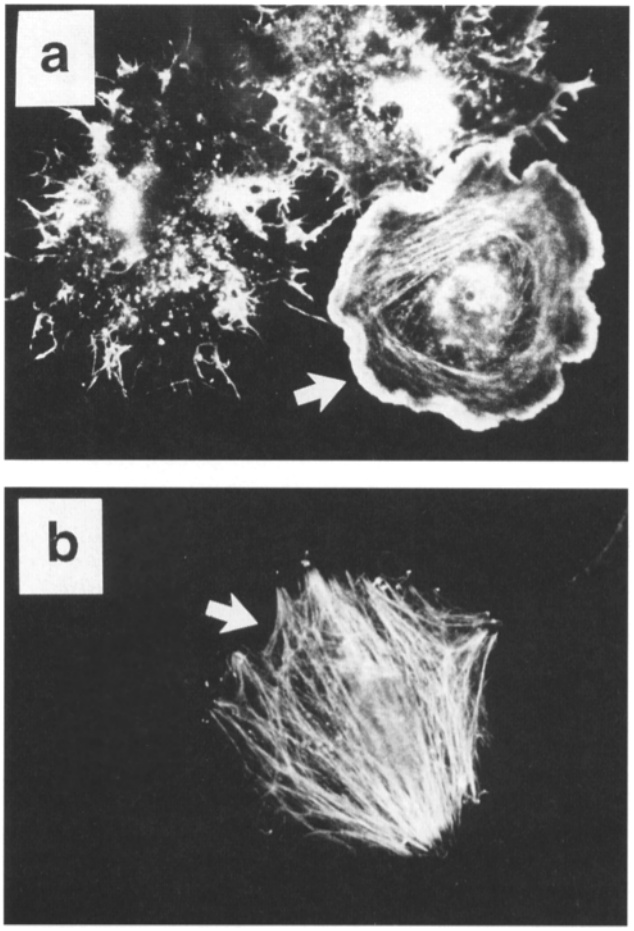

Figure 2. Rho-dependent assembly of actin fibers in cells plated on PLL and FN. Serum-starved Swiss 3T3 cells resuspended in serum-free medium were allowed to attach to coverslips coated with 10 μg/ml PLL (*a*) or 50 μg/ml FN (*b*). After 30 min cells were microinjected with recombinant rho protein, left for a further 20 min, fixed, and then stained for actin using TRITC-conjugated phalloidin. Microinjected cells are arrowed.

To determine whether focal complex assembly on fibronectin is dependent on the interaction of integrins with RGD sequences, cells were plated on FN in the presence of PDGF (5 ng/ml) or LPA (100 ng/ml) along with inhibitory (GRGDS) or noninhibitory (GRADSP) peptides (1 mM). After 40 min cells were fixed and stained for the presence of vinculin. As shown in Fig. 4, PDGF- (*b* and *e*) or LPA- (*d* and *f*) induced redistribution of vinculin to focal complexes was almost completely inhibited in the presence of GRGDS, but was unaffected by the control peptide GRADSP. As expected no vinculin-containing complexes were present in the absence of growth factors, though it was noticeable that cells plated in the presence of GRGDS peptides had a more rounded phenotype (Fig. 4, *a* and *d*).

To determine if RGD sequences are sufficient to allow focal complex formation, cells were plated on ProNectin F

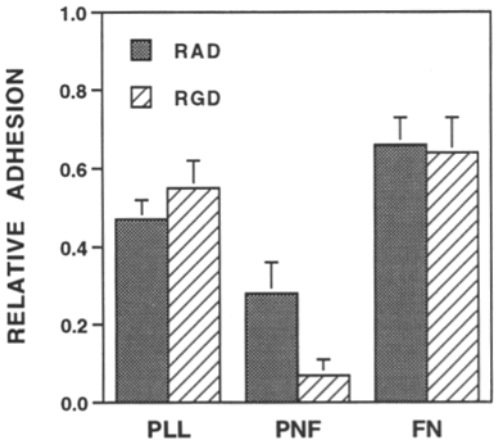

Figure 3. Effects of RGD-containing peptide on attachment of Swiss 3T3 cells to fibronectin. Serum-starved Swiss 3T3 cells were allowed to attach to microtitre wells coated with 10 μg/ml poly-L-lysine (*PLL*), 10 μg/ml ProNectin F (*PNF*), or 50 μg/ml fibronectin (*FN*) in the presence of 1 mM GRGDS (*RGD*) or GRADSP (*RAD*) peptides. Triplicate determinations were performed for each condition and the results shown here are the mean plus standard error from three separate experiments.

and then stimulated with LPA, or microinjected with recombinant rho. Focal complexes were induced under these conditions and were indistinguishable from those seen on fibronectin (data not shown).

Integrin Clustering Requires rac/rho Activity

To determine if the clustering of integrins is also dependent on functional rho/rac GTPases, we have made use of human dermal fibroblasts, since it has proved difficult to study integrins directly in Swiss 3T3 where antibody reagents useful for immunofluorescence are not readily available. When plated on VN in the presence of serum, dermal fibroblasts showed well developed vinculin-containing focal adhesions (not shown), though unlike Swiss 3T3 fibroblasts it was not possible to disassemble them completely by serum removal. As expected however, microinjection of the cells with the rho inhibitor C3 transferase, resulted in a complete loss of punctate vinculin staining within 15 min (not shown). Using an anti β3 integrin antibody, it can be seen that the clustering of integrins in focal adhesions was also completely dependent on functional rho activity since focal complex staining was lost in cells microinjected with C3 (Fig. 5 *a*, *arrowed cell*). Thus extracellular matrix alone is not sufficient to cause, or maintain, detectable clustering of surface integrins. Coinjection of C3 transferase, with a mutant rho that is resistant to C3 transferase (rac60rho; Diekmann and Hall, unpublished data), resulted in reclustering of integrins and the reassembly of focal adhesion complexes (Fig. 5 *c*, *arrowed cell*). Coinjection of C3 transferase with recombinant rac also induced clustering of integrins (Fig. 5 *b*, *arrowed cell*) and vinculin (not shown), but into small focal complexes distributed along the periphery of injected cells.

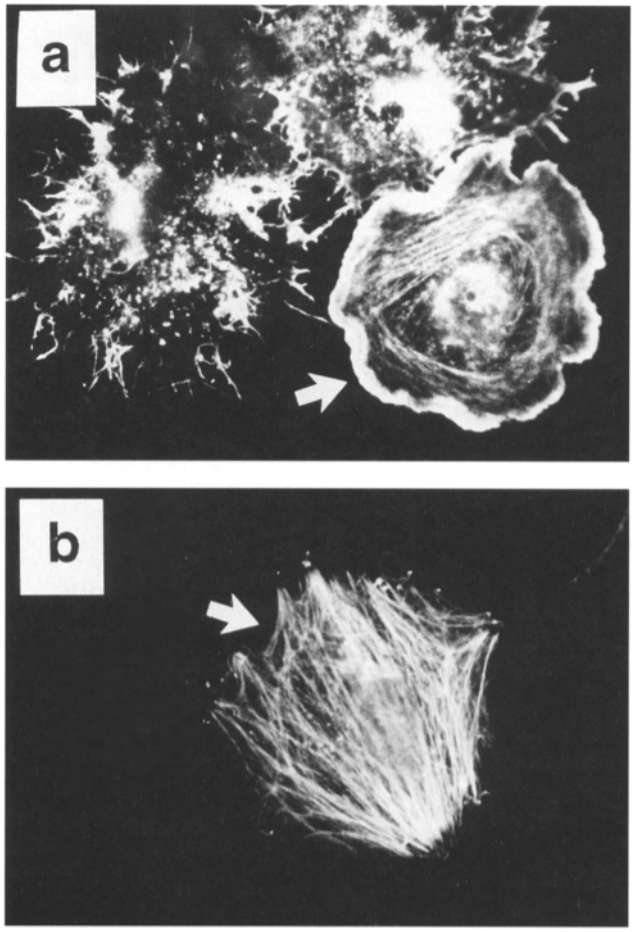

Figure 4. RGD-containing peptide inhibits rho and rac-dependent adhesion complex formation in Swiss 3T3 cells. Serum-starved cells were plated on fibronectin in the presence of control GRADSP peptide (*a–c*) or inhibitory GRGDS peptide (*d–f*) and in serum-free medium (*a* and *d*), 5 ng/ml PDGF (*b* and *e*), or 100 ng/ml LPA (*c* and *f*). Cells were allowed to attach for 40 min before being fixed and stained with an antibody to vinculin.

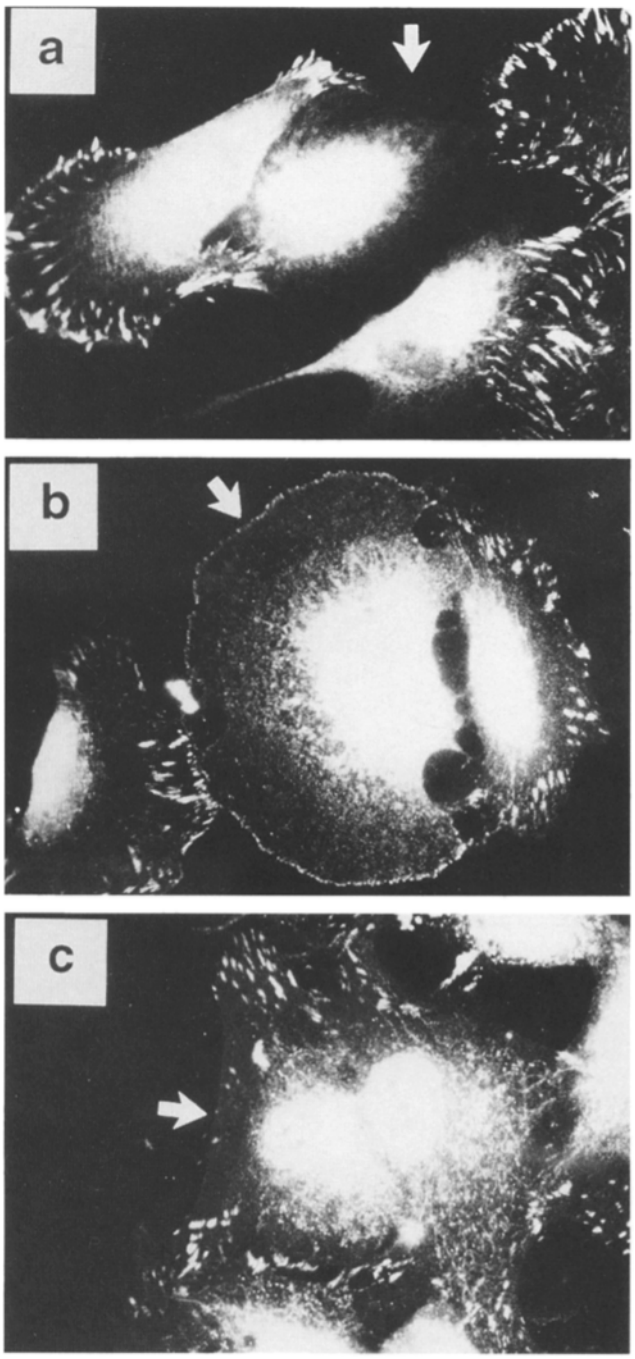

Figure 5. Redistribution of integrin into adhesion complexes is dependent on rac or rho. Human dermal fibroblasts were plated on coverslips coated with vitronectin and allowed to attach for 45 min in the presence of 10% serum. Cells were microinjected with recombinant C3 transferase (*a*), L61rac plus C3 transferase (*b*), and rac60rho plus C3 transferase (*c*). 20 min after injection cells were fixed and stained with an antibody to human β3 integrin. Microinjected cells are arrowed.

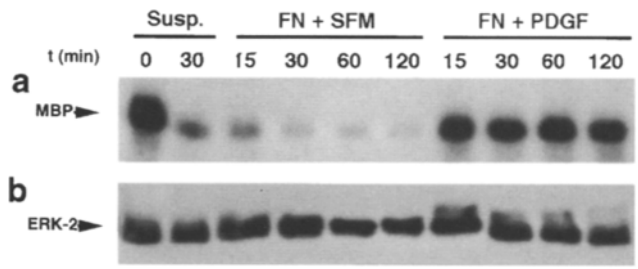

Figure 6. Fibronectin alone is insufficient to activate ERK-2 kinase. Serum-starved Swiss 3T3 cells were trypsinized and placed in suspension in dishes coated with BSA to prevent reattachment. Aliquots of cells were lysed immediately after trypsinization (lane *1*) or after 30 min in suspension (lane *2*). After 30 min cells were transferred to dishes coated with 50 μg/ml fibronectin in the presence (lanes *7–10*) or absence (lanes *3–6*) of 5 ng/ml PDGF. Adherent cells were lysed after 15, 30, 60, and 120 min and ERK-2 immunoprecipitated from the lysates. Immunoprecipitated ERK-2 was subjected to an in vitro kinase assay using myelin basic protein (*a*). As a control, identical amounts of whole lysate were Western blotted and immunoprobed using an antibody to ERK-2 (*b*). The additional band in lanes *7–10* with slightly retarded mobility relative to ERK-2 is the phosphorylated form, indicative of activation of ERK-2.

Integrin–Matrix Interactions Are Not Sufficient to Activate the MAPK Cascade

It has been suggested that integrin–matrix interactions can activate intracellular signal transduction pathways, for example the ERK1/2 MAP kinase pathway, PIP 5-kinase and the formation of 4,5-PIP_2, and specific gene transcription (Werb et al., 1989; Chen et al., 1994; Chong et al., 1994; Schlaepfer et al., 1994; Morino et al., 1995; Zhu and Assoian, 1995). Although the mechanisms of integrin signaling are not known, it is believed that it is mediated by the proteins recruited to focal complexes (Clark and Brugge, 1995). We have now established that the assembly of integrins into focal complexes requires not only an interaction with extracellular matrix, but also functionally active rho or rac GTPases. To determine whether integrin clustering, and therefore rho/rac activity, is required for integrin signaling we have looked at the activation of the MAP kinase cascade by fibronectin.

Quiescent, serum-starved Swiss 3T3 cells were removed from dishes by trypsinization and replated onto fibronectin in the presence or absence of PDGF (5 ng/ml) for various times. We observed that trypsinization itself resulted in activation of the ERK2 MAP kinase (Fig. 6 *a*, lane *1*), though this was not sustained and cells left in suspension for 30 min (Fig. 6 *a*, lane *2*) had only slightly increased kinase activity compared to quiescent monolayers. Fig. 6 *a* shows that when such cells were replated on fibronectin in the absence of growth factors (SFM) no activation of ERK2 could be detected (Fig. 6 *a*, lanes *3–6*). However, when cells were replated in the presence of PDGF, ERK2 was rapidly stimulated and this was sustained for at least 2 h (Fig. 6 *a*, lanes *7–10*).

To examine whether matrix attachment was necessary for growth factor induced activation of ERK-2 kinase, cells were trypsinized, resuspended in serum-free medium and held in suspension for 30 min to allow ERK-2 activity, induced by trypsinization to fall to background (Fig. 7 *a*, lane *1*). At this time cells were either maintained in suspension in the presence of 5 ng/ml PDGF or allowed to attach to fibronectin in the presence of PDGF. Under these conditions, substantial and sustained activation of ERK-2 was observed both in cells held in suspension (Fig. 7 *a*, lanes *2–4*) and in those plated on fibronectin (Fig. 7 *a*, lanes *5–7*).

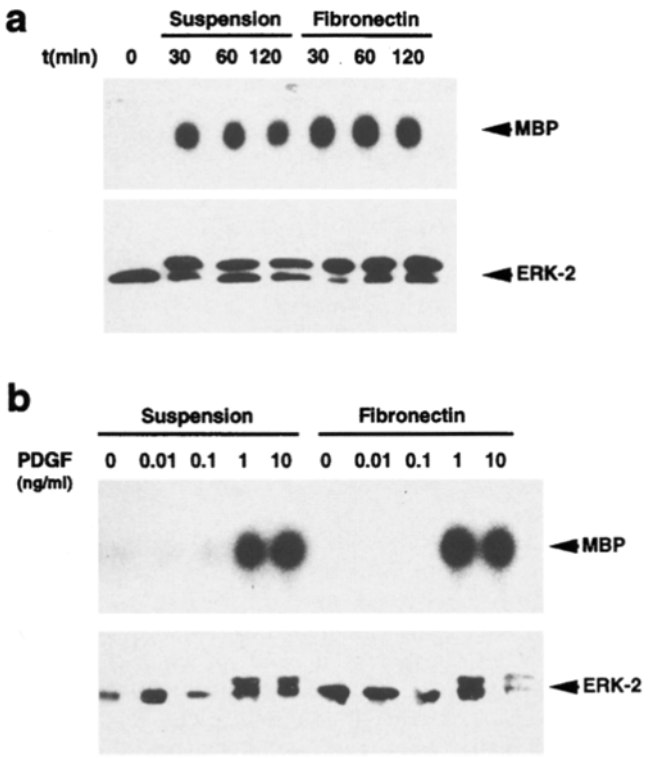

Figure 7. ERK-2 can be activated in the absence of integrin-matrix interaction. (*a*) The kinetics of ERK-2 activation in adherent and nonadherent cells was assessed by trypsinizing quiescent, serum-starved Swiss 3T3 cells, resuspending in serum-free medium and incubating at 37°C in BSA-coated dishes for 30 min to allow ERK-2 activity to return to basal levels. (lane *1*). After 30 min PDGF (5 ng/ml) was added and cells left in suspension (lanes *2–4*) or allowed to attach to dishes coated with fibronectin (lanes *5–7*). After 30, 60, and 120 min ERK-2 was immunoprecipitated and its activity towards myelin basic protein (*MBP*) determined (*top*). To control for equal loading, lysates were electrophoresed and ERK-2 levels detected by Western blotting (*bottom*). (*b*) To determine whether the dose response of adherent and nonadherent cells to PDGF was similar, cells were trypsinized and resuspended in serum-free medium for 30 min as described for *a*. After 30 min in suspension cells were left in suspension for a further 30 min in the presence of varying amounts of PDGF (lanes *1–5*) or plated on fibronectin in the presence of PDGF (lanes *6–10*). In vitro kinase assays (*MBP*) and Western blots (*ERK-2*) were performed as described for *a*.

The levels of activation observed were similar, and were comparable to that seen in quiescent, serum-starved control cultures of Swiss 3T3 cells treated with PDGF (data not shown).

To determine if there was any synergistic effect of ECM on PDGF-activation of ERK-2, cells were treated with increasing concentrations of PDGF either in suspension (Fig. 7 *b*, lanes *1–5*) or after plating on fibronectin (Fig. 7 *b*, lanes *6–10*). As can be seen, no significant difference in the dose response was observed between cells in suspension or on fibronectin with substantial activation occurring at 1 ng/ml.

Discussion

It has previously been shown that rho and rac, two members of the rho subfamily of small GTPases, are required for the formation of focal complexes in response to extracellular growth factors. Rho regulates the assembly of "classical" focal adhesion complexes, while rac controls a distinct population of focal complexes found at the leading edge of lamellipodia (Ridley and Hall, 1992; Ridley et al., 1992; Nobes and Hall, 1995). In contrast, others have suggested that the engagement of integrins by extracellular matrix is in itself sufficient to trigger the formation of focal adhesions (Woods et al., 1986, 1993; Singer et al., 1987; Miyamoto et al., 1995). We have now examined the relationship between extracellular matrix and the rho GTPase family in more detail using Swiss 3T3 fibroblasts and human dermal fibroblasts. In the absence of rho or rac activity, Swiss 3T3 cells plated on fibronectin do not assemble vinculin-containing focal complexes. Similarly, activation of rho or rac in cells plated on a poly-L-lysine substrate does not result in the assembly of focal complexes. We conclude, therefore, that formation of focal complexes is dependent on two signals, one provided by the interaction of extracellular matrix with integrins and the second provided intracellularly by functionally active members of the rho family. The requirement for a second signal in addition to an RGD–integrin interaction has been suggested previously by others, though it was thought that this was provided by other motifs in fibronectin (Woods et al., 1986, 1993; Huhtala et al., 1995). In the cases where matrix has been reported to be sufficient for focal adhesion assembly, the role of the rho GTPases has not been addressed. It is worth noting, for example, that in many cell types (Swiss 3T3 being a prominent exception) simply removing serum is not sufficient to down-regulate rho activity (our unpublished data). In some cells the basal rho activity may be intrinsically high, or perhaps more likely, maintained by autocrine production of growth factors.

The biochemical mechanism of assembly of focal complexes, which are large clusters of integrins and associated cytoplasmic-derived proteins, is not known (Burridge et al., 1988; Turner and Burridge, 1991). It has been reported that in human endothelial cells plated on fibronectin or vitronectin, clustering of integrin receptors occurs before recruitment of vinculin, while others found that talin accumulated into adhesion complexes before vinculin suggesting a sequential recruitment of components (Dejana et al., 1988; DePasquale and Izzard, 1991). Using EM techniques, aggregates of β1 integrin have been observed in the absence of vinculin or talin, in Xenopus fibroblasts and these "primordial" integrin clusters do not appear to be associated with actin structures (Samuelsson et al., 1993).

More recently, using antibody- and ligand-coated beads, it has been reported that focal complex assembly requires both integrin–ligand interaction and aggregation of integrins (Miyamoto et al., 1995). It was proposed that clustering of integrins is a direct consequence of binding to multivalent ligand. Our results, whilst supporting the hypothesis that focal complex formation requires aggregation of integrins, indicate that integrin clustering is a consequence of rho and rac activities rather than simply binding to a multivalent ligand. Although we cannot address from our experiments whether there is any order in which the complexes are assembled, the rate determining step must be regulated by rho GTPases. One possibility is that rho

GTPases direct clustering of integrins, by modifying their cytoplasmic tails, and that this then allows other proteins of the adhesion complex to assemble. An alternative possibility for in vivo assembly, is that the GTPases lead to modification of one or more of the components of the focal complex. Since many of these components as well as integrin cytoplasmic tails have been shown to interact (albeit weakly) in vitro with each other this could lead to clustering through the formation of multiple cross-linking interactions (Turner and Burridge, 1991; Sastry and Horwitz, 1993; Schaller and Parsons, 1994; Gilmore and Burridge, 1995).

Although the nature of these putative modifications is not known, we have previously reported that rho-induced focal adhesion assembly is blocked by staurosporine, suggesting that a kinase is involved (Nobes and Hall, 1995). In addition there is evidence to suggest that a genistein-sensitive tyrosine kinase is involved downstream of rho (Ridley and Hall, 1994; Nobes et al., 1995). This would be in agreement with the findings of others that assembly of focal complexes can be inhibited by tyrosine kinase inhibitors (Burridge et al., 1992; Chrzanowska-Wodnicka and Burridge, 1994). Although the downstream effectors of rho remain to be identified, one obvious candidate is the Focal Adhesion Kinase, pp125^{FAK} (FAK). FAK is a nonreceptor tyrosine kinase, which is present in focal complexes and is known to phosphorylate other focal complex components including paxillin and tensin (Schaller and Parsons, 1994). Furthermore, phosphorylation of FAK is increased when quiescent serum-starved Swiss 3T3 cells are treated with LPA (Barry and Critchley, 1994; Chrzanowska-Wodnicka and Burridge, 1994; Ridley and Hall, 1994). Whatever the modification induced by rho, it must be dynamic, since in both Swiss 3T3 cells and human dermal fibroblasts inhibiting rho leads to disassembly of focal adhesions within 5–10 min (Ridley and Hall, 1992; Nobes and Hall, 1995).

Focal complexes are found intimately associated with the actin cytoskeleton, but its role in complex assembly is unclear. It has been reported that cytochalasin inhibits both stress fiber and focal adhesion formation induced by growth factors, but when activated rho is microinjected into cells treated with cytochalasin D apparently normal focal adhesions can be observed in the absence of stress fibers (Nobes and Hall, 1995). The simplest explanation of these results is that cytochalasin D interferes with growth factor receptor activation of rho GTPases, in which case caution is required in attributing cytochalasin-induced effects to actin-dependent signals. We report here, that rho can induce actin fiber formation in the absence of focal adhesions suggesting that the two activities of rho, focal adhesion formation and actin polymerization, are separable discrete activities (Nobes and Hall, 1995). In agreement with this, inhibition of integrin-mediated cell-cell aggregation of B-lymphoblastoid cells is not blocked by cytochalasin B but is blocked by the rho inhibitor C3 transferase (Tominaga et al., 1993). Although this was interpreted as rho being required for inside-out signaling (i.e., regulating the strength of integrin–ligand interactions) we believe that it is more likely to be due to inhibition of rho-dependent clustering of integrins.

Cellular adhesion through integrins has been shown to affect a wide variety of cellular processes; including cell migration, proliferation, differentiation, and mitosis (Hynes, 1992; Adams and Watt, 1993; Clark and Brugge, 1995). In the absence of matrix, for example, fibroblasts will not grow, and arrest in late G1 of the cell cycle (Guadagno and Assoian, 1991). Transformation of cells with oncogenes overcomes this "anchorage-dependent" phenotype (Shin et al., 1975). It has been suggested that the G1 block may be due to a lack of transcription of the cyclin A gene in the absence of matrix, but the intracellular signal transduction pathways mediated by integrin engagement are unclear (Guadagno et al., 1993; Hansen et al., 1994). It has recently been reported that FAK can interact with Grb2, an adaptor protein for the ras exchange factor, Sos (Schlaepfer et al., 1994). Furthermore, several groups have reported that plating cells on fibronectin causes stimulation of ERK1/2 MAP kinase activity (Chen et al., 1994; Schlaepfer et al., 1994; Morino et al., 1995; Zhu and Assoian, 1995). In light of our observations that two signals are required for clustering of integrins into focal complexes, we examined whether rho or rac activity are also required for integrin signaling. Using quiescent Swiss 3T3 cells, in which rho and rac have been switched off by serum starvation, we find that fibronectin is unable to stimulate significant, detectable ERK2 activity.

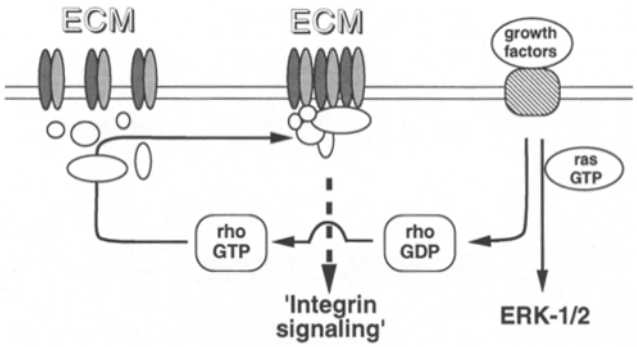

Figure 8. Model summarizing the role for rho-family GTPases and extracellular matrix in focal complex assembly and integrin-dependent signaling events.

In conclusion, attachment of cells to ECM is not sufficient to induce clustering of integrins, focal complex formation and subsequent activation of intracellular signaling pathways, but requires, in addition, functionally active intracellular rho-family GTPases (Fig. 8). Since the MAP kinase, ERK-2, can be activated in suspension, and in the absence of matrix, by PDGF, it is likely that the ras/MAP kinase signaling pathway is not an anchorage-dependent signal required for G1 progression and growth. Further analysis of the ECM-dependence of other signal transduction pathways will be required to identify these signals.

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