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Dissociation of Mitogen-activated Protein Kinase Activation from p125 Focal Adhesion Kinase Tyrosine Phosphorylation in Swiss 3T3 Cells Stimulated by Bombesin, Lysophosphatidic Acid, and Platelet-derived Growth Factor

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The experiments presented here were designed to examine the contribution of p125 focal adhesion kinase ($p125^{FAK}$) tyrosine phosphorylation to the activation of the mitogen-activated protein kinase cascade induced by bombesin, lysophosphatidic acid (LPA), and platelet-derived growth factor (PDGF) in Swiss 3T3 cells. We found that tyrosine phosphorylation of $p125^{FAK}$ in response to these growth factors is completely abolished in cells treated with cytochalasin D or in cells that were suspended in serum-free medium for 30 min. In marked contrast, the activation of $p42^{mapk}$ by these factors was independent of the integrity of the actin cytoskeleton and of the interaction of the cells with the extracellular matrix. The protein kinase C inhibitor GF 109203X and down-regulation of protein kinase C by prolonged pretreatment of cells with phorbol esters blocked bombesin-stimulated activation of $p42^{mapk}$, $p90^{rsk}$, and MAPK kinase-1 but did not prevent bombesin-induced tyrosine phosphorylation of $p125^{FAK}$. Furthermore, LPA-induced $p42^{mapk}$ activation involved a pertussis toxin-sensitive guanylate nucleotide-binding protein, whereas tyrosine phosphorylation of $p125^{FAK}$ in response to LPA was not prevented by pretreatment with pertussis toxin. Finally, PDGF induced maximum $p42^{mapk}$ activation at concentrations (30 ng/ml) that failed to induce tyrosine phosphorylation of $p125^{FAK}$. Thus, our results demonstrate that $p42^{mapk}$ activation in response to bombesin, LPA, and PDGF can be dissociated from $p125^{FAK}$ tyrosine phosphorylation in Swiss 3T3 cells.

INTRODUCTION

The mitogen-activated protein kinases (MAPKs) are a family of highly conserved serine/threonine kinases that are activated by a range of extracellular signals (Davis, 1993; Marshall, 1995). The two best characterized isoforms $p42^{mapk}$ and $p44^{mapk}$ are directly activated by phosphorylation on specific tyrosine and threonine residues by the dual-specificity MAPK kinase (or MEK), of which at least two isoforms have been identified in mammalian cells (Payne *et al.*, 1991; Crews *et al.*, 1992; Wu *et al.*, 1993). Several pathways leading to MEK activation have been demonstrated. Tyrosine kinase receptors induce MAPK activation via son of sevenless (SOS)-mediated accumulation of $p21^{ras}$-GTP, which then activates a kinase cascade comprising $p74^{raf-1}$, MEK, and MAPK (Davis, 1993; Marshall, 1995). The mechanisms by which guanylate nucleotide-binding protein (G protein)-coupled receptors mediate MAPK activation are less well defined, although $p21^{ras}$- and protein kinase C (PKC)-dependent pathways have been described (Howe and Marshall, 1993; Crespo, 1994; Seufferlein and Rozengurt, 1995a; Seufferlein *et al.*, 1995; Van Biesen *et al.*, 1996). In

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addition, the activation of the MAPK cascade mediated by certain G protein-coupled receptors in fibroblasts involves an as yet unidentified tyrosine kinase (Van Corven *et al.*, 1993; Hordijk *et al.*, 1994; Hawes *et al.*, 1995; Touhara *et al.*, 1995).

Integrin engagement with fibronectin leads to an increase in tyrosine phosphorylation and tyrosine kinase activity of the cytosolic focal adhesion protein-tyrosine kinase $p125^{FAK}$ (Burridge, 1992; Guan and Shalloway, 1992; Hanks *et al.*, 1992; Kornberg *et al.*, 1992; Schaller *et al.*, 1992). Recently, it has been reported that adhesion of NIH 3T3 fibroblasts to fibronectin can activate the MAPK cascade via formation of a complex of tyrosine phosphorylated $p125^{FAK}$ with GRB2, an adapter protein for the $p21^{ras}$ exchange factor SOS (Schlaepfer *et al.*, 1994). These results were supported by data demonstrating that integrin-mediated stimulation of MAPKs is, like tyrosine phosphorylation of $p125^{FAK}$, critically dependent on the integrity of the actin cytoskeleton (Chen *et al.*, 1994; Morino *et al.*, 1995; Zhu and Assoian, 1995). Thus, $p125^{FAK}$ could play a role in assembling a signaling complex that leads to the activation of the MAPK cascade. However, these findings have recently been challenged by a report demonstrating that the interaction of integrins with the extracellular matrix alone was not sufficient to induce activation of MAPKs in Swiss 3T3 cells (Hotchin and Hall, 1995).

Tyrosine phosphorylation of the cytosolic protein kinase $p125^{FAK}$ has recently been identified as an early event in the action of diverse signaling molecules that mediate cell growth and differentiation including mitogenic neuropeptides such as bombesin (Zachary *et al.*, 1992; Sinnett-Smith *et al.*, 1993; Zachary *et al.*, 1993), polypeptide growth factors such as platelet-derived growth factor (PDGF; Rankin and Rozengurt, 1994), and the bioactive lipids lysophosphatidic acid (LPA), sphingosine, and sphingosylphosphorylcholine (Seufferlein and Rozengurt, 1994a,b; Seufferlein and Rozengurt, 1995b). The increase in tyrosine phosphorylation of $p125^{FAK}$ is accompanied by profound alterations in the organization of the actin cytoskeleton and the integrity of the actin cytoskeleton is crucial for $p125^{FAK}$ tyrosine phosphorylation (Sinnett-Smith *et al.*, 1993; Rankin and Rozengurt, 1994; Seufferlein and Rozengurt, 1994a,b; Seufferlein and Rozengurt, 1995b). The rapidity of $p125^{FAK}$ tyrosine phosphorylation induced by bombesin, LPA, or PDGF is consistent with $p125^{FAK}$ functioning in a tyrosine kinase pathway. However, the precise role of $p125^{FAK}$ tyrosine phosphorylation in the activation of MAPKs induced by growth factors remains poorly understood.

Here, we examined the potential contribution of tyrosine-phosphorylated $p125^{FAK}$ in the activation of the MAPK cascade induced by bombesin, LPA, and PDGF in Swiss 3T3 cells. Treatment of the cells with cytochalasin D completely prevented tyrosine phosphorylation of $p125^{FAK}$ in response to bombesin, LPA, and PDGF, but did not affect $p42^{mapk}$ activation by these growth factors. Furthermore, bombesin, LPA, and PDGF stimulated $p42^{mapk}$ activation but did not induce $p125^{FAK}$ tyrosine phosphorylation in cells kept in suspension. Thus, our results dissociate $p42^{mapk}$ activation from $p125^{FAK}$ tyrosine phosphorylation in growth factor-stimulated Swiss 3T3 cells.

MATERIALS AND METHODS

Cell Culture

Stock cultures of Swiss 3T3 fibroblasts were maintained in DMEM supplemented with 10% fetal bovine serum in a humidified atmosphere containing 10% CO_2 and 90% air at 37°C. For experimental purposes, cells were plated either in 35-mm Nunc Petri dishes at 10^5 cells/dish or in 100-mm dishes at 6×10^5 cells/dish in DMEM containing 10% fetal bovine serum and used after 6–8 days when the cells were confluent and quiescent. For assays using suspended cells, confluent and quiescent Swiss 3T3 cells in 100-mm dishes were washed three times with DMEM and subsequently suspended in 10 ml of DMEM by gentle scraping. The cell suspension was then incubated for 30 min at 37°C before stimulation with the respective factors.

Immunoprecipitations of Phosphotyrosyl Proteins

Confluent and quiescent cultures of Swiss 3T3 cells in 100-mm dishes or in suspension were treated with factors as indicated and lysed at 4°C in 1 ml of 10 mM Tris/HCl (pH 7.6), 5 mM EDTA, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM NaF, 100 μM Na_3VO_4, 1% Triton X-100, and 1 mM phenylmethylsulfonyl fluoride (lysis buffer). Lysates of these cells were clarified by centrifugation at $15,000 \times g$ for 10 min. $p125^{FAK}$ was immunoprecipitated overnight at 4°C with agarose-linked monoclonal antibody (mAb) 2A7. Immunoprecipitates were washed twice with lysis buffer, extracted in 2× sample buffer (200 mM Tris-HCl, 6% SDS, 2 mM EDTA, 4% 2-mercaptoethanol, 10% glycerol, pH 6.8), and then resolved by one-dimensional SDS-PAGE before autoradiography.

Western Blotting

After SDS-PAGE, proteins were transferred to Immobilon transfer membranes. For detection of tyrosine-phosphorylated proteins, membranes were blocked using 3% nonfat dried milk in phosphate-buffered saline (pH 7.2) and incubated for 2 h with antiphosphotyrosine [anti-Tyr(P)] mAbs (Py20 and 4G10, 1 μg/ml each) in phosphate-buffered saline containing 3% nonfat dried milk. Immunoreactive bands were visualized by autoradiography using ^{125}I-labeled sheep anti-mouse IgG (1 μCi/ml). Expression of cyclins D1, D3, and E, retinoblastoma (Rb), and $p27^{kip1}$ was determined by Western blotting essentially as described above using specific antisera with immunoreactive bands being visualized using horseradish peroxidase-conjugated anti-rabbit IgG and subsequent enhanced chemiluminescence detection.

p42mapk Mobility Shift Assays

Activation of $p42^{mapk}$ can be determined by the appearance of slower migrating forms in SDS-PAGE gels, which results from the phosphorylation of specific threonine and tyrosine residues within its subdomain VIII (Leevers and Marshall, 1992). Quiescent cultures of Swiss 3T3 cells were treated with factors as indicated: the cells were lysed in 2× SDS-PAGE sample buffer and analyzed by SDS-PAGE. Proteins were subsequently transferred to Immobilon mem-

branes which were blocked as described above and incubated for 1 h at 22°C with a polyclonal p42^{mapk} antiserum (1:1000) in phosphate-buffered saline containing 3% nonfat dried milk. Immunoreactive bands were visualized using ^{125}I-labeled protein A followed by autoradiography.

Immune Complex Kinase Assays for p42^{mapk} and p90^{rsk} Activation

Quiescent and confluent Swiss 3T3 cells in 100-mm dishes or in suspension were treated with factors as described in the figure legends and lysed at 4°C. Lysates were clarified by centrifugation at 15,000 × *g* for 10 min at 4°C. Immunoprecipitation was performed using a polyclonal anti-p42^{mapk} antibody or a polyclonal anti-p90^{rsk} antibody and incubating the samples on a rotating wheel for 2 h. Protein A-agarose beads (40 μl, 1:1 slurry) were added for the second hour. Immune complexes were collected by centrifugation and washed twice in lysis buffer and twice in kinase buffer (15 mM Tris-HCl, pH 7.4, 15 mM $MgCl_2$). The kinase reaction was performed by resuspending the pellet in 25 μl of kinase assay cocktail containing kinase buffer, 100 μM ATP, 100 μCi/ml [γ-^{32}P] ATP, 200 μM microcystin LR, and either 1 mg/ml myelin basic protein-peptide (APRTPGGRR) or S6 peptide (RRRLSSLRA) for the assays of p42^{mapk} and p90^{rsk}, respectively. Incubations were performed for 10 min under linear assay conditions at 30°C and terminated by spotting 20 μl of the supernatant onto P81 chromatography paper (Whatman). Filters were washed four times for 5 min in 0.5% *o*-phosphoric acid, immersed in acetone, and dried before Cerenkov counting. The average radioactivity of two blank samples containing no immune complex was subtracted from the result of each sample. Results are expressed as cpm/1.5 × 10^6 cells. The specific activity of [γ-^{32}P]ATP used was 900-1200 cpm/pmol.

MEK-1 Kinase Assay

MEK-1 activity was assayed with a modification of the method described by Alessi *et al.* (1994) using a fusion protein of p42^{mapk} as substrate. Overnight cultures of *Escherichia coli* strain BL21 DE3 transformed with glutathione *S*-transferase (GST)-p42^{mapk} expression vectors (pGEX-2T) were diluted 1:10 and grown for 1 h. GST-p42^{mapk} was induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside for 4 h at 37°C. The cells were then pelleted, freeze thawed, and lysed. Cell debris was removed by centrifugation, and the GST fusion protein was purified by adding 0.5 ml of glutathione-Sepharose beads and rotating for 30 min at 4°C. The GST-p42^{mapk} was subsequently cleaved from the GST in thrombin buffer, dialyzed, and concentrated. The purity of the preparation was checked by subjecting the protein to SDS-PAGE and staining the gels with Coomassie blue. Typical yields for GST-p42^{mapk} were 10 μg/ml of culture, with a purity of >95%. For the kinase assays, quiescent cells were treated as indicated and lysed in lysis buffer as described above with the addition of 100 nM microcystin LR, 10 μg/ml aprotinin, and 10 μg/ml leupeptin. Immunoprecipitations were performed by incubating the lysates with a mAb anti-MEK-1 antibody for 2 h, with 40 μl of protein A-agarose (1:1 slurry) added for the second hour. Immune complexes were collected by centrifugation and washed three times in lysis buffer without phenylmethylsulfonyl fluoride and once with buffer A (50 mM Tris-HCl, pH 7.5, 0.1 mM EGTA, 0.5 mM Na_3VO_4, 0.1% 2-mercaptoethanol). Pellets were then resuspended in 30 μl of MAPK buffer (30 mM Tris-HCl, pH 7.5, 0.1 mM EGTA, 0.1% 2-mercaptoethanol, 100 μg/l GST-MAPK, 0.03% Brij-35, 10 mM Mg-ATP, 20 mM *N*-octyl-β-D-glucopyranoside) and incubated at 30°C for 30 min. The reaction was then terminated by diluting the supernatant in 40 μl of buffer A containing 1 mg/ml of bovine serum albumin, and, after mixing, 10 μl of the supernatant were removed to a fresh tube. MAPK activation was then measured using the myelin basic protein-peptide phosphorylation assay essentially as described above. The specific activity of [γ-^{32}P]ATP used was 900-1200 cpm/pmol.

Materials

Bombesin, LPA, and cytochalasin D were obtained from Sigma. The PKC inhibitor GF 109203X, thapsigargin, and microcystin LR were obtained from Calbiochem-Novabiochem (Nottingham, United Kingdom). Enhanced chemiluminescence reagent, PDGF (BB homodimer), ^{125}I-labeled protein A (15 mCi/mg), ^{125}I-labeled sheep anti-mouse IgG (15 mCi/mg), and [γ-^{32}P]ATP (370 MBq/ml) were obtained from Amersham (Amersham, United Kingdom). PY 20 anti-Tyr(P) mAb, 4G10 anti-Tyr(P) mAb, and mAb 2A7 directed against p125^{FAK} were obtained from United Biomedical (Lake Placid, NY). The polyclonal anti-p42^{mapk} antibody raised against a COOH-terminal peptide (EETARFQPGYRS) and the polyclonal antibody against p90^{rsk} raised against a COOH-terminal peptide (IESSILAQRRVRKLPSTTL) were a generous gift from Dr. J. Van Lint (Katholieke Universiteit Leuven, Belgium). The monoclonal anti-MEK-1 antibody and the monoclonal anti-p125^{FAK} antibody for Western blotting were from Affiniti Research (Nottingham, United Kingdom). The anti-p27^{kip1} and anti-cyclin D3 and E antibodies were obtained Santa Cruz Biotechnology (Santa Cruz, CA). The anti-Rb antibody was obtained from PharMingen and the anti-cyclin D1 antibody was a kind gift from Dr. G. Peters (Imperial Cancer Research Fund). The GST-p42^{mapk} expression vector was a generous gift from Professor C. Marshall (Institute of Cancer Research, London, United Kingdom). All other reagents were of the purest grade available.

RESULTS

Effect of Cytochalasin D on Bombesin-induced p125^{FAK} Tyrosine Phosphorylation and Activation of p42^{mapk}

Tyrosine phosphorylation of p125^{FAK} in response to growth factors is critically dependent on the integrity of the actin cytoskeleton (Sinnett-Smith *et al.*, 1993; Rankin and Rozengurt, 1994; Seufferlein and Rozengurt, 1994a). To examine whether the marked increase in p125^{FAK} tyrosine phosphorylation induced by bombesin (Zachary and Rozengurt, 1992; Sinnett-Smith *et al.*, 1993) was involved in bombesin-induced p42^{mapk} activation, quiescent cultures of Swiss 3T3 cells were treated for 2 h with increasing concentrations of cytochalasin D, which selectively disrupts the network of the actin filaments. The cells were subsequently stimulated with 10 nM bombesin for 5 min, and p125^{FAK} tyrosine phosphorylation and p42^{mapk} activation were determined by immunoblotting anti-p125^{FAK} immunoprecipitates using an anti-Tyr(P) mAb and by mobility shift and immune complex kinase assays, respectively. As shown in Figure 1A (filled circles), cytochalasin D prevented bombesin-induced p125^{FAK} tyrosine phosphorylation in a concentration-dependent manner; a complete inhibition was achieved at 1.2 μM. In contrast, cytochalasin D at various concentrations did not inhibit p42^{mapk} activation in response to bombesin as judged by both the mobility shift and immune complex kinase assays (Figure 1A, top and bottom panels, open circles). Cytochalasin D at 2 μM also did not inhibit p42^{mapk} activation in response to various concentrations of bombesin (0.03–10 nM; Figure 1B). Furthermore, treat-

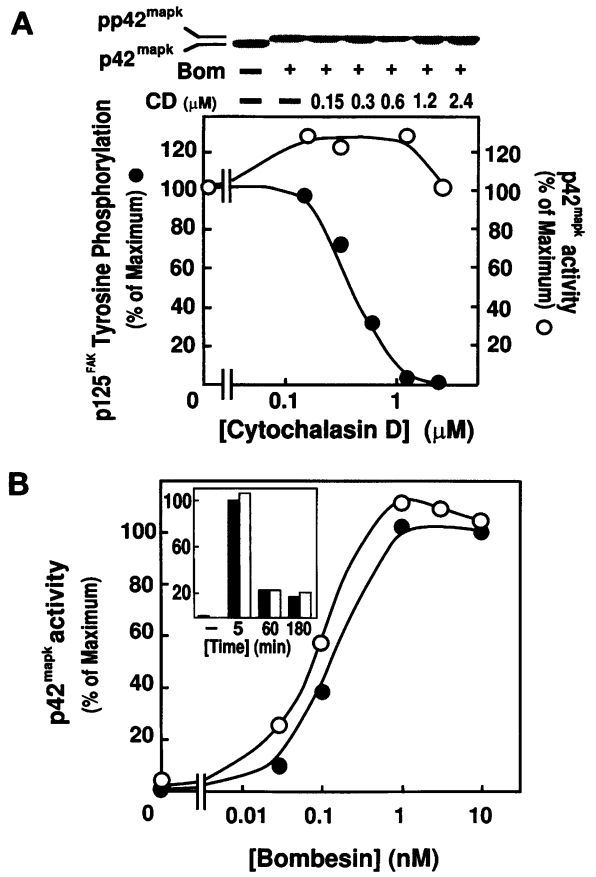


Figure 1. Effect of cytochalasin D on bombesin-stimulated $p125^{FAK}$ tyrosine phosphorylation and $p42^{mapk}$ activation. (A) Quiescent Swiss 3T3 cells were washed and incubated for 2 h at 37°C with various concentrations of cytochalasin D (CD) or received an equivalent amount of solvent (−). Top panel: the cells were subsequently stimulated with 10 nM bombesin (Bom) for 5 min and lysed. The lysates were then subjected to SDS-PAGE followed by Western blotting with anti-$p42^{mapk}$ antibody. The results shown are representative of three independent experiments. The positions of nonphosphorylated $p42^{mapk}$ and the slower migrating phosphorylated form of $p42^{mapk}$ are indicated. Bottom panel: cells were treated with cytochalasin D and stimulated with bombesin as described above and lysed in lysis buffer, and lysates were either immunoprecipitated with anti-$p42^{mapk}$ polyclonal antibody followed by immune complex kinase assays (open circles) or with mAb 2A7 directed against $p125^{FAK}$ followed by Western blotting with anti-Tyr(P) mAbs (filled circles). Results are representative of three independent experiments. The results of the immune complex kinase assays are the means of duplicates and are expressed as percentages of the maximum of bombesin-stimulated $p42^{mapk}$ activation (5000–6000 cpm/1.5 × 10^6 cells at 5 min). The increase in tyrosine phosphorylation of $p125^{FAK}$ was quantified by scanning densitometry, and values are expressed as percentages of the maximum increase in tyrosine phosphorylation above unstimulated control values. (B) Quiescent Swiss 3T3 cells were treated with 2 μM cytochalasin D for 2 h (open circles) or received an equivalent amount of solvent (filled circles). The cells were subsequently incubated with various concentrations of bombesin for 5 min, lysed, and immune complex kinase assays were performed using anti-$p42^{mapk}$ polyclonal antibody as described in MATERIALS AND METHODS. Results are expressed as percentages of the maximum of bombesin-stimulated activation (5000–6000 cpm/1.5 × 10^6 cells at 5 min), and the results shown are representative of three independent experiments. Inset: Quiescent Swiss 3T3 cells were incubated with 2 μM cytochalasin D (open columns) for 2 h or received an equivalent amount of solvent (filled columns). The cells were subsequently stimulated with 10 nM bombesin for various times as indicated and lysed in lysis buffer, and lysates were immunoprecipitated with anti-$p42^{mapk}$ polyclonal antibody followed by immune complex kinase assays. The results are the means of duplicates expressed as a percentage of the maximum of bombesin-stimulated $p42^{mapk}$ activation (5000–6000 cpm/1.5 × 10^6 cells at 5 min) and are representative of three independent experiments.

ment with 2 μM cytochalasin D did not have any effect on either the early (5 min) or the sustained phase (60 and 180 min) of bombesin-induced $p42^{mapk}$ activation (Figure 1B, inset).

In contrast to the results obtained with cytochalasin D, pretreatment of Swiss 3T3 cells with increasing concentrations of the PKC inhibitor GF 109203X for 1 h prevented the subsequent activation of $p42^{mapk}$ in response to bombesin in a concentration-dependent manner (Figure 2A, open circles). A complete inhibition of bombesin-induced $p42^{mapk}$ activation was observed at 5 μM GF 109203X. However, tyrosine phosphorylation of $p125^{FAK}$ in response to bombesin was virtually unaffected by treatment of parallel cultures of Swiss 3T3 cells with various concentrations of GF 109203X (Figure 2A, filled circles). Similarly, down-regulation of phorbol ester-sensitive PKCs by chronic pretreatment of the cells with phorbol 12,13-dibutyrate (PDB) markedly inhibited bombesin-induced $p42^{mapk}$ activation (Figure 2A, inset, open columns), whereas $p125^{FAK}$ tyrosine phosphorylation in response to bombesin was not affected by this treatment (Figure 2A, inset, filled columns).

To further substantiate these results, we examined the effect of cytochalasin D and GF 109203X on bombesin-stimulated activation of $p90^{rsk}$, a major downstream target of $p42^{mapk}$ (Sturgill and Wu, 1991; Blenis, 1993). As shown in Figure 2B, treatment of Swiss 3T3 cells with 2 μM cytochalasin D did not interfere with bombesin-stimulated activation of $p90^{rsk}$. In contrast, pretreatment of Swiss 3T3 cells with GF 109203X or down-regulation of PKC by prolonged pretreatment with PDB largely prevented $p90^{rsk}$ activation in response to bombesin (Figure 2B).

MEK-1 is a major upstream regulator of bombesin-induced $p42^{mapk}$ activation in Swiss 3T3 cells (Seufferlein *et al.*, 1996). Activation of MEK-1 by bombesin was not affected by treatment of the cells with cytochalasin D. However, bombesin-induced activation of MEK-1 was completely prevented in GF 109203X-treated cells (Figure 2C).

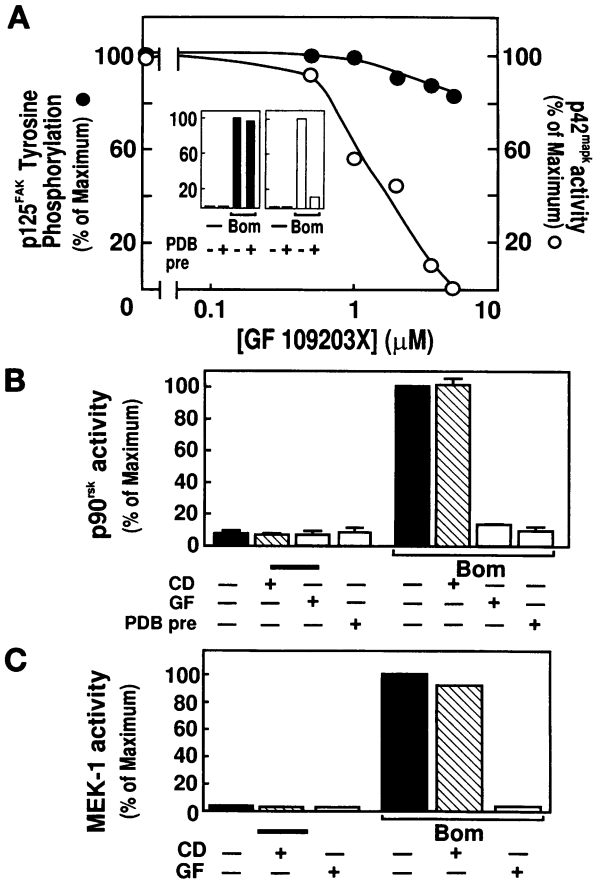
Thus, activation of the MAPK cascade in response to bombesin can be dissociated from tyrosine phosphorylation of $p125^{FAK}$ in Swiss 3T3 cells.

Effect of Cytochalasin D on PDGF- and LPA-induced Tyrosine Phosphorylation of p125^{FAK} and Activation of p42^{mapk}

Next, we examined whether tyrosine phosphorylation of p125^{FAK} could be involved in MAPK activation by LPA and PDGF which, unlike bombesin (Mitchell *et al.*, 1995), stimulate the MAPK cascade in a p21^{ras}-dependent manner (Van Corven *et al.*, 1993; Hordijk *et al.*, 1994; Marshall, 1995).

LPA potently induces tyrosine phosphorylation of p125^{FAK} (Kumagai *et al.*, 1993; Seufferlein and Rozengurt, 1994a), and this effect could be prevented by pretreatment of the cells with cytochalasin D (Figure 3A, top panel). As shown in Figure 3A (bottom panel), cytochalasin D did not affect LPA-induced activation of p42^{mapk} in parallel cultures. LPA stimulates p42^{mapk} activation by a pathway dependent on the function of a pertussis toxin-sensitive heterotrimeric G protein of the G_0-G_i subfamily (Van Corven *et al.*, 1993; Hordijk *et al.*, 1994). Pretreatment of Swiss 3T3 cells with pertussis toxin (30 ng/ml for 3h) failed to prevent LPA-induced tyrosine phosphorylation of p125^{FAK}. In contrast, p42^{mapk} activation in response to LPA was inhibited by 75% in parallel cultures (Figure 3B). This suggests that different G proteins mediate the effect of LPA on p125^{FAK} tyrosine phosphorylation and p42^{mapk} activation, respectively.

PDGF acts on receptors that possess an intrinsic tyrosine kinase activity (Heldin, 1992; Claesson-Welsh, 1994). Recently, it has been described that PDGF at low concentrations (5 ng/ml) can stimulate tyrosine phosphorylation of p125^{FAK} (Rankin and Rozengurt, 1994). Treatment of Swiss 3T3 cells with 2 μM cytochalasin D completely prevented tyrosine phosphorylation of p125^{FAK} in response to PDGF at 5 ng/ml (Figure 4A, top panel). However, cytochalasin D did not inhibit p42^{mapk} activation in response to 5 ng/ml of PDGF (Figure 4A, bottom panel). Recent



(Figure 2 cont.) stimulated with 10 nM bombesin for 5 min and lysed, and the lysates were immunoprecipitated with mAb 2A7 directed against p125^{FAK} followed by Western blotting with anti-Tyr(P) mAbs (filled columns) or with anti-p42^{mapk} polyclonal antibody followed by immune complex kinase assays (open columns). The results of the immune complex kinase assays are the means of duplicates, expressed as percentages of the maximum of bombesin-stimulated p42^{mapk} activation (5000–6000 cpm/1.5 × 10^6 cells at 5 min), and representative of two independent experiments. The increase in tyrosine phosphorylation of p125^{FAK} was quantified by scanning densitometry, and values are expressed as percentages of the maximum increase in tyrosine phosphorylation above unstimulated control values. (B) Quiescent Swiss 3T3 cells were incubated with 2 μM cytochalasin D for 2 h (CD +) or 3.5 μM GF 109203X for 1 h (GF +), or were pretreated with 800 nM PDB for 48 h to down-regulate PKC (PDB pre +). Control cells received an equivalent amount of solvent (−). Cells were subsequently stimulated with 10 nM bombesin for 5 min and then lysed, and the lysates were further analyzed by performing p90^{rsk} immune complex kinase assays as described in MATERIALS AND METHODS. Results are the means ± SE of three independent experiments, each performed in duplicate, and expressed as percentages of the maximum of bombesin-stimulated p90^{rsk} activity (10,000–15,000 cpm/1.5 × 10^6 cells at 5 min). (C) Quiescent Swiss 3T3 cells were incubated with 2 μM cytochalasin D for 2 h (CD +) or 3.5 μM GF 109203X for 1 h (GF +), or received an equivalent amount of solvent (−) and were subsequently stimulated with 10 nM bombesin for 3 min. The cells were lysed and the lysates further analyzed by performing MEK-1 kinase assays as described in MATERIALS AND METHODS. Results are the means of two independent experiments, each performed in duplicate, and expressed as percentages of the maximum of bombesin-stimulated MEK-1 activity (19,000–25,000 cpm/1.5 × 10^6 cells at 3 min).

Figure 2. Role of PKC in bombesin-stimulated p125^{FAK} tyrosine phosphorylation and activation of p42^{mapk}, p90^{rsk}, and MEK-1. (A) Quiescent Swiss 3T3 cells were washed and incubated for 1 h at 37°C with various concentrations of the selective PKC inhibitor GF 109203X. The cells were subsequently stimulated with 10 nM bombesin for 5 min and lysed, and the lysates were either immunoprecipitated with anti-p42^{mapk} polyclonal antibody followed by immune complex kinase assays (open circles) or with mAb 2A7 directed against p125^{FAK} followed by Western blotting with anti-Tyr(P) mAbs (filled circles). Results are representative of three independent experiments. The results of the immune complex kinase assays are the means of duplicates and are expressed as percentages of the maximum of bombesin-stimulated p42^{mapk} activation (5000–6000 cpm/1.5 × 10^6 cells at 5 min). The increase in tyrosine phosphorylation of p125^{FAK} was quantified by scanning densitometry and values are expressed as percentages of the maximum increase in tyrosine phosphorylation above unstimulated control values. Inset: Quiescent Swiss 3T3 cells were incubated with 800 nM PDB for 48 h to down-regulate PKC (PDB pre +). Control cells received an equivalent amount of solvent (−). The cells were subsequently

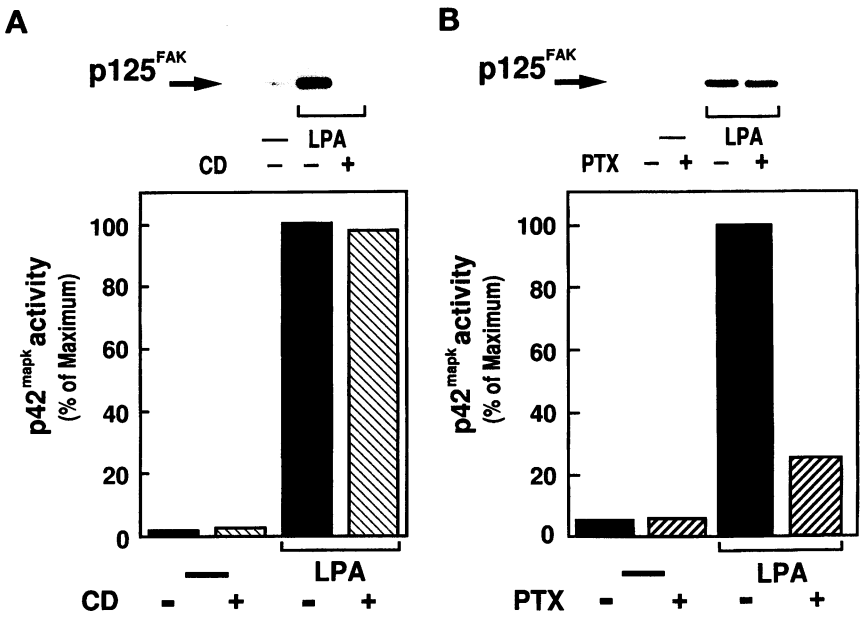


Figure 3. Effect of cytochalasin D and pertussis toxin on LPA-induced p125^{FAK} tyrosine phosphorylation and activation of p42^{mapk}. (A) Quiescent Swiss 3T3 cells were washed and incubated for 2 h at 37°C with 2 μM cytochalasin D (CD +) or received an equivalent amount of solvent (−). Top panel: the cells were subsequently stimulated with 2 μM LPA for 5 min and lysed. The lysates were then immunoprecipitated with mAb 2A7 directed against p125^{FAK} followed by Western blotting with anti-Tyr(P) mAbs. The results shown are representative of three independent experiments. The position of p125^{FAK} is indicated by an arrow. Bottom panel, cells were treated with cytochalasin D and stimulated with LPA as described above, lysed in lysis buffer, and the lysates were immunoprecipitated with anti-p42^{mapk} polyclonal antibody followed by immune complex kinase assays. Results are representative of three independent experiments. The results of the immune complex kinase assays are the means of duplicates and expressed as percentages of the maximum of LPA-stimulated p42^{mapk} activation (4500–5500 cpm/1.5 × 10^6 cells at 5 min). (B) Quiescent Swiss 3T3 cells were washed and incubated for 3 h at 37°C with 30 ng/ml pertussis toxin (PTX +) or received an equivalent amount of solvent (−). Top panel, the cells were subsequently stimulated with 2 μM LPA for 5 min and lysed; the lysates were then immunoprecipitated with mAb 2A7 directed against p125^{FAK} followed by Western blotting with anti-Tyr(P) mAbs. The results shown are representative of three independent experiments. The position of p125^{FAK} is indicated by an arrow. Bottom panel, cells were treated with pertussis toxin and stimulated with LPA as described above, lysed in lysis buffer, and the lysates were immunoprecipitated with anti-p42^{mapk} polyclonal antibody followed by immune complex kinase assays. Results are representative of three independent experiments. The results of the immune complex kinase assays are the means of duplicates and expressed as percentages of the maximum of LPA-stimulated p42^{mapk} activation (4500–5500 cpm/1.5 × 10^6 cells at 5 min).

data from our laboratory have demonstrated that PDGF at a high concentration (30 ng/ml) can abolish bombesin- or LPA-induced actin stress fiber formation and inhibit tyrosine phosphorylation of p125^{FAK} (Rankin and Rozengurt, 1994; Seufferlein and Rozengurt, 1994a). Accordingly, PDGF at 30 ng/ml failed to induce tyrosine phosphorylation of p125^{FAK} (Figure 4B, top panel). In contrast, PDGF at 30 ng/ml still induced maximum activation of p42^{mapk} in Swiss 3T3 cells (Figure 4B, bottom panel). In agreement with these results, pretreatment of the cells with 2 μM cytochalasin D did not prevent p42^{mapk} activation in response to 30 ng/ml od PDGF (Figure 4B, inset).

Role of Adhesion-dependent Signals in p125^{FAK} Tyrosine Phosphorylation and in the Activation of p42^{mapk} by Bombesin, LPA, and PDGF

To examine the role of adhesion-dependent signals in tyrosine phosphorylation of p125^{FAK} and p42^{mapk} activation in response to bombesin, LPA, and PDGF, parallel cultures of adherent and suspended Swiss 3T3 cells were stimulated with bombesin, LPA, or PDGF for 5 min. The cells were subsequently lysed, and the lysates were further analyzed for p42^{mapk} activation or tyrosine phosphorylation of p125^{FAK} as described in MATERIALS AND METHODS. As shown in Figure 5A (top panel), tyrosine phosphorylation of p125^{FAK} in response to bombesin, LPA, and PDGF was completely prevented in cells kept in suspension for 30 min. The protein levels of p125^{FAK} were comparable in the samples of suspended and adherent cells as demonstrated by Western blotting with anti-p125^{FAK} mAb (Figure 5A, top panel). In marked contrast, the levels of p42^{mapk} activation by bombesin, LPA, and PDGF in parallel cultures of suspended cells were comparable to that obtained in adherent Swiss 3T3 cells (Figure 5A, bottom panel).

The kinetics of p42^{mapk} activation by bombesin in Swiss 3T3 cells kept in suspension closely resembled that in parallel quiescent adherent cultures with an early peak in kinase activity 5 min after stimulation, followed by a sustained phase of activation reaching 20% of the maximum after 60 min of incubation with bombesin (Figure 5B, left panel). In addition, treatment of suspended cells with 3.5 μM GF 109203X markedly inhibited p42^{mapk} activation in response to bombesin (our unpublished results). We also examined the time courses of p42^{mapk} activation in adherent and suspended Swiss 3T3 cells stimulated by either PDGF or LPA. As shown in Figure 5B (middle panel),

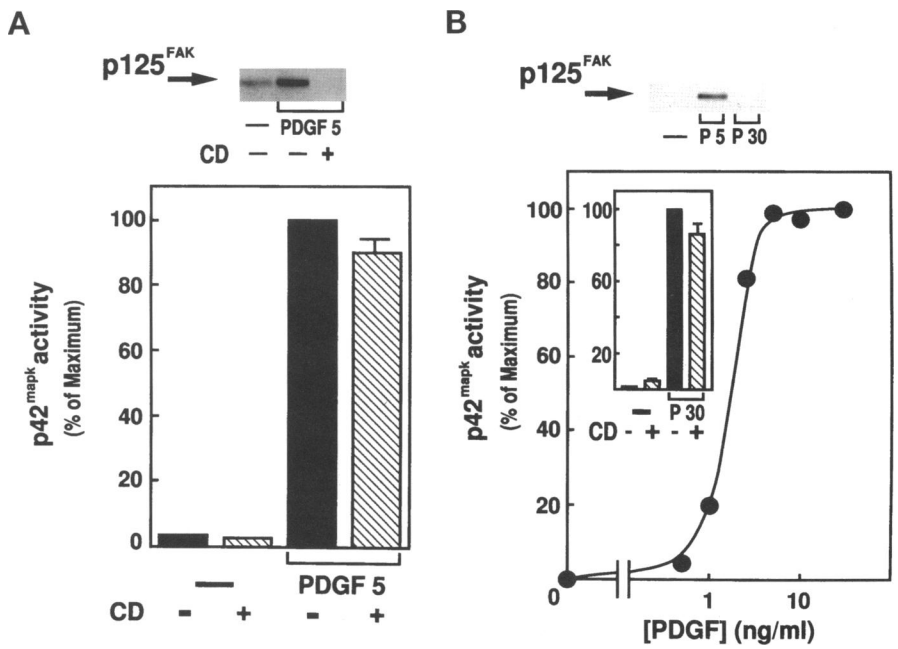


Figure 4. Effect of cytochalasin D on PDGF-induced p125^{FAK} tyrosine phosphorylation and activation of p42^{mapk}. (A) Swiss 3T3 cells were washed and incubated for 2 h at 37°C with 2 μM cytochalasin D (CD +) or received an equivalent amount of solvent (−). Top panel, the cells were subsequently stimulated with 5 ng/ml PDGF (PDGF 5) for 5 min and lysed; the lysates were then immunoprecipitated with mAb 2A7 directed against p125^{FAK} followed by Western blotting with anti-Tyr(P) mAbs. The results shown are representative of three independent experiments. The position of p125^{FAK} is indicated by an arrow. Bottom panel, cells were treated with cytochalasin D and stimulated with 5 ng/ml PDGF as described above, lysed in lysis buffer, and the lysates were immunoprecipitated with anti-p42^{mapk} polyclonal antibody followed by immune complex kinase assays. Results are representative of three independent experiments. The results of the immune complex kinase assays are the means of duplicates and expressed as percentages of the maximum of PDGF-stimulated p42^{mapk} activation (6500–7500 cpm/1.5 × 10^6 cells at 5 min). (B) Top panel, Swiss 3T3 cells were stimulated with 5 ng/ml of PDGF (P 5) or 30 ng/ml of PDGF (P 30) for 5 min and lysed; the lysates were then immunoprecipitated with mAb 2A7 directed against p125^{FAK} followed by Western blotting with anti-Tyr(P) mAbs. The results shown are representative of three independent experiments. Bottom panel, Swiss 3T3 cells were incubated with various concentrations of PDGF for 5 min as indicated, lysed in lysis buffer, and the lysates were immunoprecipitated with anti-p42^{mapk} polyclonal antibody followed by immune complex kinase assays. The results shown are the means of duplicates expressed as percentages of the maximum of PDGF-stimulated p42^{mapk} activation (6500–7500 cpm/1.5 × 10^6 cells at 5 min) and are representative of two independent experiments. Inset, cells were treated with 2 μM cytochalasin D for 2 h (CD +) or received an equivalent amount of solvent (−) and were subsequently stimulated with 30 ng/ml of PDGF for 5 min, lysed, and the lysates were immunoprecipitated with anti-p42^{mapk} polyclonal antibody followed by immune complex kinase assays as described in MATERIALS AND METHODS. Results are representative of three independent experiments. The results of the immune complex kinase assays are the means of duplicates and expressed as percentages of the maximum of PDGF-stimulated p42^{mapk} activation (6500–7500 cpm/1.5 × 10^6 cells at 5 min).

PDGF induced an early peak in p42^{mapk} activity followed by a sustained phase of activation in both adherent and suspended cells. In contrast, LPA stimulated a transient activation of p42^{mapk} in both adherent or suspended cells but did not cause a sustained activation of this kinase (Figure 5B, right panel). Thus, in contrast to tyrosine phosphorylation of p125^{FAK}, activation of p42^{mapk} by bombesin, LPA, and PDGF is anchorage independent.

Effect of Cytochalasin D on Cyclin D and E Expression, Elimination of p27^{kip-1}, and Hyperphosphorylation of Rb

The results presented here demonstrate that cytochalasin D, at concentrations that completely block the increase in tyrosine phosphorylation of p125^{FAK}, does not interfere with activation of p42^{mapk} and p90^{rsk} in response to bombesin. In other cell types, cytochalasin D inhibits the transition of quiescent cells into the S phase of the cell cycle at the R point in late G_1 (Böhmer *et al.*, 1996). Passage through the R point is regulated by the coordinated accumulation of cyclins D and E which bind to and activate cyclin-dependent kinase (cdk) subunits (Sherr, 1993) and by the elimination of the cdk inhibitor p27^{kip1} (Sherr and Roberts, 1995). Rb has been identified as a substrate of cyclin D1 activity in late G_1. Consequently, we examined the effect of cytochalasin D on bombesin-induced expression of cyclins D and E, elimination of p27^{kip1}, and phosphorylation of Rb. Similar experiments were performed with Swiss 3T3 cells exposed to PDGF.

Stimulation of Swiss 3T3 cells with either bombesin or PDGF caused a marked increase in the expression of cyclins D1 and D3 and in the phosphorylation of Rb, as judged by the electrophoretic mobility shift of this protein (Figure 6.) Bombesin and PDGF also induced cyclin E expression and down-regulation of p27^{kip1}. Treatment with cytochalasin D profoundly inhibited the expression of cyclins D1, D3, and E as well as the hyperphosphorylation of Rb. In contrast, cytochalasin D, which alone reduced the level of p27^{kip1}, did not prevent elimination of this protein in response to bombesin or PDGF. Our results indicate that cytochalasin D inhibits the passage through the R

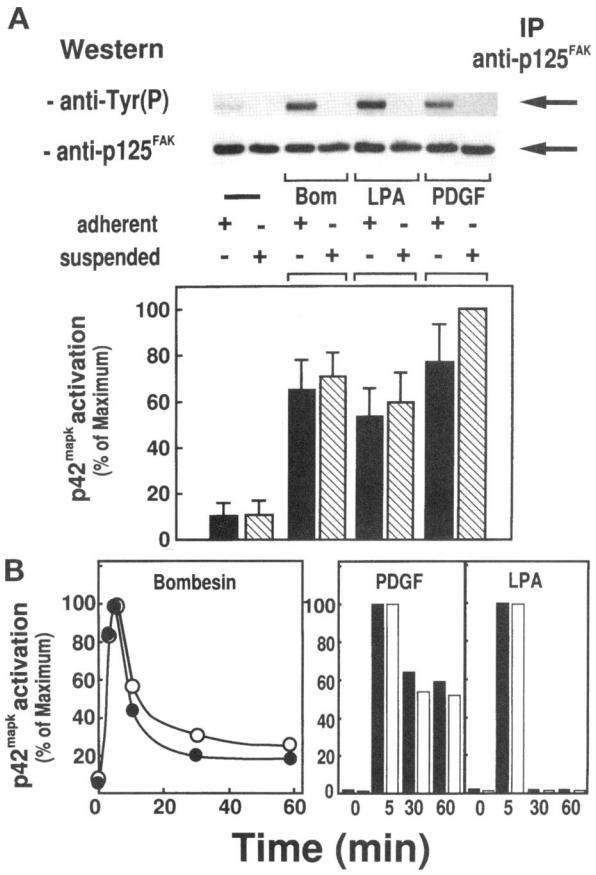


Figure 5. Role of adhesion-dependent signals in bombesin-, LPA-, and PDGF-induced tyrosine phosphorylation of $p125^{FAK}$ and activation of $p42^{mapk}$. (A) Top panel, quiescent Swiss 3T3 cells in 100-mm dishes (adherent) or Swiss 3T3 cells suspended in DMEM for 30 min were stimulated with either 10 nM bombesin (Bom), 2 μM LPA, or 5 ng/ml of PDGF for 5 min. The cells were subsequently lysed and the lysates were immunoprecipitated with mAb 2A7 directed against $p125^{FAK}$ followed by Western blotting with anti-Tyr(P) mAbs [anti-Tyr(P)]. The membrane was subsequently reprobed with a mAb directed against $p125^{FAK}$ (anti-$p125^{FAK}$). Results are representative of three independent experiments. Bottom panel, parallel cultures of quiescent Swiss 3T3 cells in 100-mm dishes (adherent) or Swiss 3T3 cells suspended in DMEM for 30 min were stimulated with either 10 nM bombesin (Bom), 2 μM LPA, or 5 ng/ml of PDGF for 5 min. The cells were subsequently lysed and the lysates were immunoprecipitated with anti-$p42^{mapk}$ polyclonal antibody followed by immune complex kinase assays as described in MATERIALS AND METHODS. Results are the means ± SE of three independent experiments, each performed in duplicate, and expressed as percentages of the maximum of PDGF-stimulated $p42^{mapk}$ activation obtained in suspended Swiss 3T3 cells (7000–8000 cpm/1.5 × 10^6 cells at 5 min). (B) Parallel cultures of Swiss 3T3 cells, either adherent (filled circles and columns) or suspended in DMEM for 30 min (open circles and columns), were stimulated with either 10 nM bombesin (left panel), 5 ng/ml of PDGF (middle panel), or 2 μM LPA (right panel) for various times as indicated and lysed, and the lysates were immunoprecipitated with anti-$p42^{mapk}$ polyclonal antibody followed by immune complex kinase assays. The results are representative of three independent experiments, the means of duplicates, and expressed as percentages of the maximum of $p42^{mapk}$ activation (bombesin, 5000–6000 cpm/1.5 × 10^6 cells at 5 min; PDGF, 6500–7500 cpm/1.5 × 10^6 cells at 5 min; LPA, 4500–5500 cpm/1.5 × 10^6 cells at 5 min).

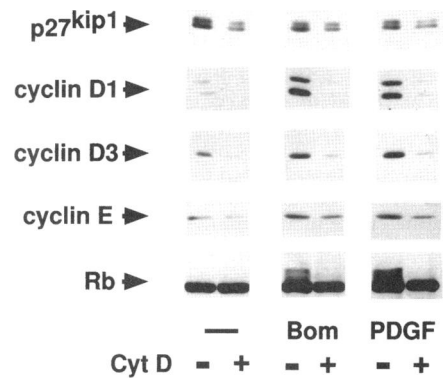


Figure 6. Effect of cytochalasin D on cyclin D and E expression, elimination of $p27^{kip1}$, and hyperphosphorylation of Rb. Confluent and quiescent cultures of Swiss 3T3 cells were washed and incubated at 37°C in 2 ml of DMEM/Waymouth's medium with either vehicle (−), 10 nM bombesin (Bom), or 5 ng/ml of PDGF either with (+) or without (−) 1 h preincubation with 2 μM cytochalasin D (Cyt D). After 15 h cells were lysed and Western blotting was performed for $p27^{kip1}$, cyclins D1, D3, and E, and Rb as described in MATERIALS AND METHODS. Results presented are typical of three independent experiments.

point of the cell cycle in Swiss 3T3 cells stimulated by bombesin or PDGF.

DISCUSSION

Engagement of integrins has recently been demonstrated to promote $p125^{FAK}$ tyrosine phosphorylation and subsequent association of tyrosine-phosphorylated $p125^{FAK}$ with the GRB2/SOS complex, leading to activation of $p21^{ras}$ and subsequently $p42^{mapk}$ in NIH 3T3 cells (Schlaepfer *et al.*, 1994). Furthermore, several groups have reported that plating cells on fibronectin causes activation of $p42^{mapk}$ and that this effect depends on the integrity of the actin cytoskeleton (Chen *et al.*, 1994; Morino *et al.*, 1995). Growth factors such as bombesin, LPA, and PDGF potently stimulate tyrosine phosphorylation of $p125^{FAK}$ (Kumagai *et al.*, 1993; Sinnett-Smith *et al.*, 1993; Rankin and Rozengurt, 1994; Seufferlein and Rozengurt, 1994a). However, the precise contribution, if any, of $p125^{FAK}$ tyrosine phosphorylation to the stimulation of the MAPK cascade induced by growth factors, as opposed to integrins, has not been explored. In the present study, we examined whether tyrosine-phosphorylated $p125^{FAK}$ is an upstream regulator of $p42^{mapk}$ activation in bombesin-, LPA-, or PDGF-stimulated Swiss 3T3 cells.

Bombesin, LPA, and PDGF induce tyrosine phosphorylation of $p125^{FAK}$ through pathways that are critically dependent on the organization of the actin cytoskeleton (Sinnett-Smith *et al.*, 1993; Rankin and Rozengurt, 1994; Seufferlein and Rozengurt, 1994a). In this report, we have shown that disruption of the cytoskeleton by cytochalasin D at concentrations that

completely prevented tyrosine phosphorylation of p125^{FAK} in response to bombesin, LPA, and PDGF did not interfere with p42^{mapk} activation by these growth factors. The results imply that cytochalasin D dissociates MAPK activation from p125^{FAK} tyrosine phosphorylation in growth factor-stimulated Swiss 3T3 cells. Dissolution of actin filaments can also be induced by preventing integrin-mediated organization of the actin cytoskeleton, i.e., by suspending cells in serum-free medium. To substantiate our results obtained with cytochalasin D, we also examined p125^{FAK} tyrosine phosphorylation and p42^{mapk} activation in response to bombesin, LPA, and PDGF in suspended cells. We found that these growth factors failed to induce p125^{FAK} tyrosine phosphorylation in suspended cultures of Swiss 3T3 cells. In marked contrast, activation of the MAPK cascade by LPA, bombesin, or PDGF was completely anchorage independent. Indeed, our results show that the kinetics of p42^{mapk} activation induced by bombesin, PDGF, and LPA in cells kept in suspension are comparable to those in adherent cultures of Swiss 3T3 cells.

The results obtained with suspended cells not only confirm that p42^{mapk} activation by bombesin, LPA, and PDGF is independent of p125^{FAK} tyrosine phosphorylation but also have another interesting implication. It has been suggested that sustained activation of p42/p44^{mapk} is both necessary and sufficient to induce proliferation or differentiation of various cell lines (Meloche *et al.*, 1992; Pages *et al.*, 1993). However, in the absence of interaction with the extracellular matrix or in the presence of cytochalasin D, fibroblasts are known to arrest in late G_1 (Guadagno and Assoian, 1991; Guadagno *et al.*, 1993; Böhmer *et al.*, 1996; Fang *et al.*, 1996). Consequently, activation of the MAPK cascade by growth factors in the absence of adhesion-dependent signals as shown in the present study is not sufficient to trigger the progression of Swiss 3T3 fibroblasts from G_0-G_1 to the S phase. Interestingly, Böhmer *et al.* (1996) recently found that growth factors can also induce c-myc expression in suspended cells, indicating that various early events associated with exit from G_0 can be stimulated in an anchorage-independent manner. In contrast, the dramatic inhibition of bombesin-, LPA- and PDGF-induced p125^{FAK} tyrosine phosphorylation observed in suspended or cytochalasin D-treated cells raises the intriguing possibility that p125^{FAK} tyrosine phosphorylation could play a role in promoting molecular events in the late stage of G_1. Here, we show that treatment with cytochalasin D profoundly inhibited the expression of cyclins D1, D3, and E and hyperphosphorylation of Rb induced by either bombesin or PDGF. Recently, Böhmer *et al.* (1996) have also shown that cytochalasin D prevents the expression of cyclin D1 induced by serum in human fibroblasts, and Fang *et al.* (1996) demonstrated that the activation of the cyclin E/cdk2 complex requires adhesion-dependent signals. Taken together, these findings strongly suggest that passage through the R point requires the integrity of the actin cytoskeleton and the assembly of focal adhesion plaques. The possibility that tyrosine phosphorylation of p125^{FAK} or other focal adhesion proteins could play a role in the regulation of events in late G_1 requires additional experimental work.

We also examined the putative relationship between p42^{mapk} activation and p125^{FAK} tyrosine phosphorylation using a different approach. PKC has recently been identified as the major upstream regulator of MEK-1, p42^{mapk}, and p90^{rsk} activation in bombesin-stimulated Swiss 3T3 cells (Seufferlein *et al.*, 1996). Accordingly, we found that the selective PKC inhibitor GF 109203X potently inhibited activation of these kinases by bombesin. In contrast, at identical concentrations, GF 109203X did not prevent p125^{FAK} tyrosine phosphorylation in response to this neuropeptide. Therefore, these results also lead to the conclusion that bombesin induces tyrosine phosphorylation of p125^{FAK} and activation of MAPK by independent pathways.

LPA-stimulated p42^{mapk} activation involves a pertussis toxin-sensitive heterotrimeric G protein. In contrast, p125^{FAK} tyrosine phosphorylation by LPA is not affected by treatment of the cells with pertussis toxin, suggesting that LPA induces tyrosine phosphorylation of p125^{FAK} and activation of p42^{mapk} by activating distinct G proteins. Several reports demonstrate that certain G protein-coupled receptors such as the LPA receptor and the α2A adrenergic receptor induce p21^{ras} and MAPK activation via a tyrosine kinase pathway as inferred from the use of tyrosine kinase inhibitors (Van Corven *et al.*, 1993; Hordijk *et al.*, 1994; Hawes *et al.*, 1995; Touhara *et al.*, 1995). Our findings with cytochalasin D and suspended cells indicate that p125^{FAK} is not the putative upstream tyrosine kinase which leads to MAPK activation in Swiss 3T3 fibroblasts.

Recently, it has been described that PDGF modulation of tyrosine phosphorylation of p125^{FAK}, as with the actin cytoskeleton, is sharply dependent on PDGF concentration (Rankin and Rozengurt, 1994). Tyrosine phosphorylation of p125^{FAK} was markedly stimulated in response to low concentrations of PDGF (5 ng/ml). At higher concentrations of PDGF (30 ng/ml), p125^{FAK} tyrosine phosphorylation was dramatically decreased, resulting in a bell-shaped dose-response relationship (Rankin and Rozengurt, 1994). In contrast, p42^{mapk} activation by PDGF exhibited a sigmoidal dose-response curve which did not decrease at high concentrations when p125^{FAK} tyrosine phosphorylation was completely inhibited.

Our results demonstrate that activation of the MAPK cascade by bombesin, LPA, or PDGF can be dissociated from a signaling pathway initiated by ty-

rosine phosphorylation of p125^{FAK} in Swiss 3T3 fibroblasts.

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