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Integrins induce activation of EGF receptor: role in MAP kinase induction and adhesion-dependent cell survival

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Adhesion of human primary skin fibroblasts and ECV304 endothelial cells to immobilized matrix proteins, β1 or αv integrin antibodies stimulates tyrosine phosphorylation of the epidermal growth factor (EGF) receptor. This tyrosine phosphorylation is transiently induced, reaching maximal levels 30 min after adhesion, and it occurs in the absence of receptor ligands. Similar results were observed with EGF receptor-transfected NIH-3T3 cells. Use of a kinase-negative EGF receptor mutant demonstrates that the integrin-stimulated tyrosine phosphorylation is due to activation of the receptor's intrinsic kinase activity. Integrin-mediated EGF receptor activation leads to Erk-1/MAP kinase induction, as shown by treatment with the specific inhibitor tyrphostin AG1478 and by expression of a dominant-negative EGF receptor mutant. EGF receptor and Erk-1/MAP kinase activation by integrins does not lead *per se* to cell proliferation, but is important for entry into S phase in response to EGF or serum. EGF receptor activation is also required for extracellular matrix-mediated cell survival. Adhesion-dependent MAP kinase activation and survival are regulated through EGF receptor activation in cells expressing this molecule above a threshold level (5×10^3 receptors per cell). These results demonstrate that integrin-dependent EGF receptor activation is a novel signaling mechanism involved in cell survival and proliferation in response to extracellular matrix.

Keywords: adhesion-dependent cell survival/cell–matrix interaction/EGF receptor/integrin signaling/MAP kinase

Introduction

Integrins are cell surface adhesive receptors formed by α and β subunits, which bind to extracellular matrix proteins. Integrin-mediated adhesion stimulates multiple signaling pathways which modulate actin cytoskeleton organization, cell motility, cell growth and the ability of cells to escape from apoptosis. Integrin-dependent signaling pathways include Ca^{2+} influx, cytoplasmic alkalinization, potassium channel activation and tyrosine phosphorylation of cytoplasmic proteins (reviewed in Clark and Brugge, 1995). Moreover, integrin-mediated adhesion triggers activation of the mitogen-activated protein (MAP) kinases ERK-1 and ERK-2 (Chen *et al*., 1994; reviewed in Assoian, 1997; Giancotti, 1997; Howe *et al*., 1998).

Although many integrin-dependent signaling pathways have been described extensively, the molecular mechanisms by which integrins are able to trigger these events are still poorly defined. Since the cytoplasmic domains of both α and β integrin subunits are short and devoid of enzymatic activity, it is likely that interactions with transducing molecules have to take place in order to promote intracellular signaling. A potential candidate as a transducing element is represented by the tyrosine kinase p125Fak (Hanks *et al*., 1992; Schaller *et al*., 1992), which is located in focal adhesions and can interact with both structural cytoskeletal elements and signaling molecules. The N-terminal domain of p125Fak binds *in vitro* the cytoplasmic domain of β1 and β3 integrin subunits, while its C-terminal domain binds SH2 and SH3 domains of several proteins involved in focal adhesion assembly and signal transduction (for a review, see Malik and Parsons, 1996). Following integrin-mediated phosphorylation, Tyr397 becomes a high-affinity binding site for the SH2 domain of c-Src (Schaller *et al*., 1994), and this interaction is important in the regulation of cell motility (Cary *et al*., 1996). A role for p125Fak and associated proteins in focal adhesion turnover and cell motility is also indicated by the use of the dominant-negative form of p125Fak (Richardson and Parsons, 1996) and by p125Fak gene inactivation (Ilic *et al*., 1995). It has also been proposed that phosphorylated p125Fak interacts with the adaptor molecule Grb-2, leading to MAP kinase activation (Schlaepfer and Hunter, 1997).

The assembly of a transduction complex can also involve transmembrane proteins that co-operate with integrins to activate signaling pathways. In particular, it has been shown that caveolin, a membrane protein that links a variety of cell surface receptors to intracellular signaling pathways, can interact with integrins. Following cell–matrix adhesion, integrin–caveolin complexes associate with tyrosine-phosphorylated Shc, which, in turn, interacts with the Grb2–Sos complex leading to activation of the Ras–MAP kinase cascade (Wary *et al*., 1996). In addition, integrins can associate with proteins belonging to the Tetraspan family (CD9, CD63 and CD81) to modulate intracellular signaling (Berditchevski *et al*., 1997).

In addition to these molecules, growth factor receptors are candidates to co-operate with integrins in assembling the transduction machinery. Evidence for the formation of integrin and growth factor receptor macromolecular complexes has been suggested by co-clustering and immunofluorescence experiments (Plopper *et al*., 1995; Miyamoto *et al*., 1996; Sundberg and Rubin, 1996; Jones

et al., 1997) as well as by direct co-precipitation (Falcioni *et al*., 1997; Schneller *et al*., 1997). Schneller *et al*. (1997) showed for the first time that activation by its specific ligand leads to association of the platelet-derived growth factor (PDGF) β receptor with the αvβ3 integrin.

In the present study, we demonstrate that integrins can utilize the epidermal growth factor (EGF) receptor as a transducing element in the matrix-induced signaling pathways. We show in fact that integrins can induce EGF receptor tyrosine phosphorylation in the absence of EGF receptor ligands, leading to activation of a typical EGF receptor pathway that involves Shc phosphorylation and MAP kinase activation. In addition, we show that integrin-dependent EGF receptor activation is important in anchorage-dependent cell survival.

Results

Integrin-mediated adhesion induces tyrosine phosphorylation of the EGF receptor

The plating of human primary skin fibroblasts N6 and human endothelial cells ECV304 on matrix proteins in serum-free medium stimulates rapid tyrosine phosphorylation of 170 and 125/130 kDa proteins (Figure 1A, lanes 1–4). The 125/130 kDa proteins were identified as p125Fak and p130Cas, as previously described (Burridge *et al*., 1992; Defilippi *et al*., 1994; Nojima *et al*., 1995). To identify the 170 kDa protein, we used a panel of antibodies and showed that this protein was immunoprecipitated specifically by the EGF receptor antibody 2913 (Beguinot *et al*., 1986) (Figure 1A, lanes 5 and 6). Immunodepletion of EGF receptor from cell extracts resulted in the disappearance of the 170 kDa protein, indicating that EGF receptor is the only component of this tyrosine-phosphorylated band (Figure 1A, lanes 7 and 8). Tyrosine phosphorylation of EGF receptor in response to adhesion was also observed by plating NIH-3T3 cells transfected with the EGF receptor (Velu *et al*., 1989) on fibronectin-coated dishes (Figure 1A, lanes 9 and 10).

In order to test whether tyrosine phosphorylation of the EGF receptor during cell–matrix adhesion is induced specifically by integrins, we plated ECV304 cells on dishes coated with monoclonal antibodies to β1 and αv integrin subunits or with poly-L-lysine. While tyrosine phosphorylation of the EGF receptor was induced similarly in cells plated on both integrin antibodies, cells plated on poly-L-lysine did not display significant levels of receptor phosphorylation (Figure 1B). Moreover, tyrosine phosphorylation of the EGF receptor was also stimulated by antibody-induced β1 or β3 integrin clustering in ECV304 cells kept in suspension, indicating that integrin oligomerization by antibodies is sufficient to induce this event (data not shown).

To exclude the possibility that EGF receptor phosphorylation induced by integrin-mediated adhesion was dependent on autocrine production of its ligands, EGF receptor (EGFR)-transfected NIH-3T3 cells were treated with concentrated supernatants of both N6 and ECV304 cells, and analyzed for tyrosine phosphorylation of the EGF receptor. Neither N6- nor ECV304-conditioned medium induced any receptor phosphorylation (data not shown). Furthermore, no mRNAs for EGF or transforming growth factor-α (TGF-α) were detected by RT–PCR experiments in N6 or ECV304 cells (data not shown). Taken together, these findings indicate that adhesion-induced EGF receptor phosphorylation is not mediated by release of receptor ligands during the adhesive process.

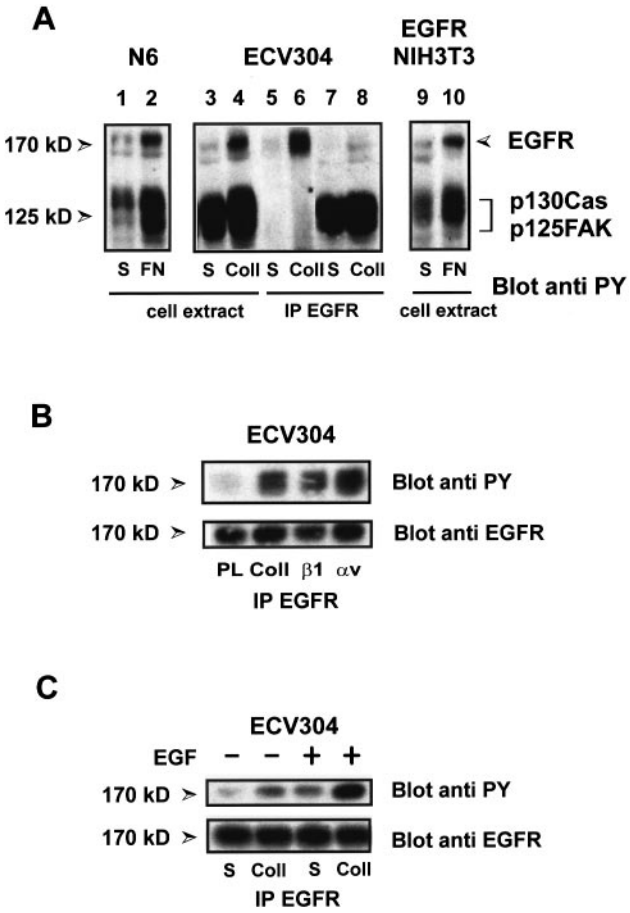

Fig. 1. Analysis of adhesion-dependent EGF receptor tyrosine phosphorylation. (**A**) N6 primary human skin fibroblasts (lanes 1 and 2), ECV304 endothelial cells (lanes 3–8) and EGFR-transfected NIH-3T3 cells (EGFR NIH3T3) (lanes 9 and 10) were serum starved, detached with EDTA and either kept in suspension (S) or allowed to adhere on dishes coated with 10 μg/ml fibronectin (FN), or collagen type I (Coll) for 30 min. Aliquots of 100 μg of cells extracts (lanes 1–4, 9 and 10) were run on 8% SDS–PAGE and immunoblotted with anti-phosphotyrosine (anti-PY) mAb PY20. A 1 mg aliquot of ECV304 cell extracts was immunoprecipitated with the EGF receptor polyclonal antibody 2913 (lanes 5 and 6), and supernatants, following two rounds of immunoprecipitations (lanes 7 and 8), were separated by SDS–PAGE and analyzed by Western blotting with anti-PY mAb PY20. The arrow on the right indicates the position of the EGF receptor; the bracket indicates the doublet constituted by p125Fak and p130Cas. (**B**) ECV304 cells were kept in suspension or plated on dishes coated with 10 μg/ml poly-L-lysine (PL), Coll, mAb BV7 to β1 integrin or mAb L230 to the αv integrin subunit. Cell extracts (1 mg) were immunoprecipitated with the EGF receptor polyclonal antibody 2913, resolved by SDS–PAGE and immunoblotted with anti-PY mAb PY20 (upper panel). After stripping, the filter was reprobed with polyclonal antibody EGFR1 (lower panel). (**C**) ECV304 endothelial cells were kept in suspension (S) or allowed to adhere on dishes coated with 10 μg/ml Coll for 30 min in the absence (–) or presence (+) of 15 ng/ml of EGF. Cell extracts were immunoprecipitated with the EGF receptor polyclonal antibody 2913, run on SDS–PAGE and immunoblotted with anti-PY mAb PY20 (upper panel). The filter was reprobed with polyclonal antibody EGFR1 (lower panel). Molecular weights are indicated on the left.

To compare the intensity of tyrosine phosphorylation of the EGF receptor induced by adhesion with that induced

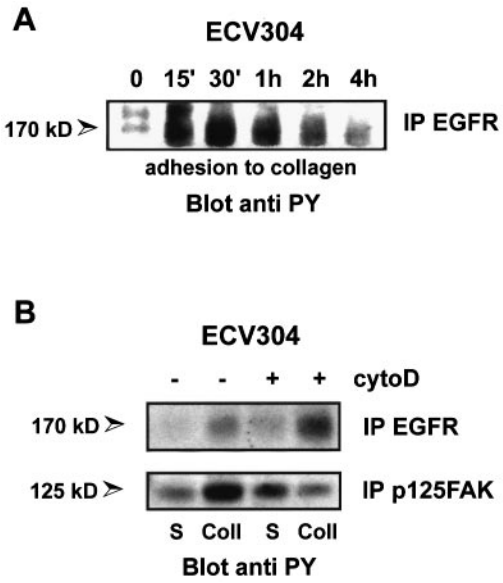

Fig. 2. Time course of EGF receptor tyrosine phosphorylation following adhesion, and effect of cytochalasin D treatment. (**A**) ECV304 endothelial cells were plated on 10 μg/ml collagen type I-coated dishes for the indicated times. Cell extracts (1 mg) were immunoprecipitated with the polyclonal antibody 2913. Immunoprecipitates were run on SDS–PAGE and immunoblotted with anti-PY mAb RC20. (**B**) Extracts of ECV304 cells kept in suspension or allowed to adhere for 30 min on 10 μg/ml collagen type I in the absence (–) or presence (+) of 0.4 μM cytochalasin D (cytoD) were immunoprecipitated with the polyclonal antibodies EGFR1 or Fak4. Tyrosine-phosphorylated EGF receptor and p125Fak were detected with anti-PY mAb RC20. A representative experiment of three is shown.

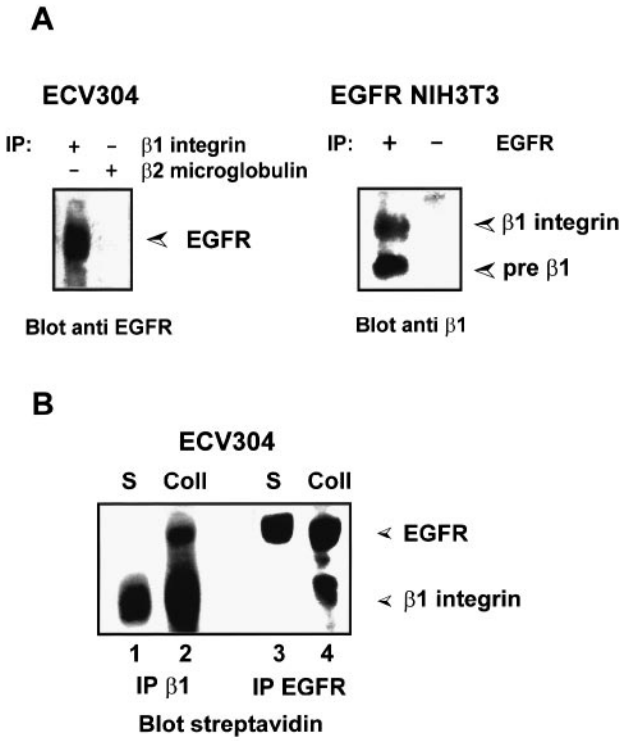

Fig. 3. Co-immunoprecipitation between EGF receptor and β1 integrin. (**A**) Cell extracts from ECV304 endothelial cells and EGFR-transfected NIH-3T3 cells were immunoprecipitated with mAb BV7 to the β1 integrin subunit, mAb RI.30 to human β_2-microglobulin, polyclonal antibody EGFR1 or pre-immune serum (–), run on SDS–PAGE, and immunoblotted with polyclonal antibody EGFR1 or with polyclonal antibody to β1 integrin. (**B**) ECV304 cells kept in suspension (S) or allowed to adhere on dishes coated with 10 μg/ml collagen were cell surface biotinylated, and extracts immunoprecipitated with mAb BV7 to the β1 integrin subunit or polyclonal antibody EGFR1 were revealed with streptavidin–HRP. The positions of the EGF receptor and β1 integrin are shown on the right.

by EGF, ECV304 cells were treated with EGF either in suspension or adherent to matrix proteins. Quantitative analysis of phosphotyrosine Western blotting indicated that adhesion-induced tyrosine phosphorylation of the EGF receptor is 5-fold less than that induced by a saturating dose of EGF (15 ng/ml) (Figure 1C, lanes 2 and 4) and similar to that obtained with 1 ng/ml EGF (data not shown). Therefore, EGF receptor tyrosine phosphorylation induced by adhesion represents a partial receptor activation, which can be increased further by addition of subsaturating concentration of EGF. In addition, the EGF receptor is phosphorylated more efficiently by the same doses of EGF in adherent cells than in cells in suspension (Figure 1D, lanes 3 and 4).

Integrin-mediated tyrosine phosphorylation of EGF receptor is transient and independent of actin cytoskeleton organization

In ECV304 cells plated on collagen type I, tyrosine phosphorylation of the EGF receptor was maximal 30 min after plating and then decreased, reaching basal levels within 4 h (Figure 2A). Phosphorylation of EGF receptor following adhesion is therefore a transient phenomenon.

Actin cytoskeleton integrity has been shown to be a major requirement in many integrin-mediated signaling events (Clark and Brugge, 1995). Therefore, ECV304 cells were plated on collagen type I in the presence of 0.4 μM cytochalasin D, a known inhibitor of actin polymerization. This treatment inhibited spreading but not adhesion on collagen. Phosphotyrosine Western blotting showed that cytochalasin D did not influence adhesion-induced tyrosine phosphorylation of the receptor (Figure 2B, upper panel), while it dramatically reduced that of p125Fak (Figure 2B, lower panel), in agreement with previous observations (Lipfert *et al*., 1992; Defilippi *et al*., 1995).

β1 integrin and EGF receptor can form a complex on the cell membrane

To investigate whether the EGF receptor and integrins associate, extracts of ECV304 cells were immunoprecipitated with β1 antibodies and blotted with EGF receptor antibodies. The EGF receptor was indeed co-immunoprecipitated with β1 integrin (Figure 3A, left panel) and the β1 integrin was detected in the anti-EGF receptor immunoprecipitate (Figure 3A, right panel), indicating that these molecules can form a complex. Immunoprecipitation of EGF receptor with β1 integrin was specific, since no co-immunoprecipitation of EGF receptor was observed with a control antibody against β_2-microglobulin or pre-immune serum (Figure 3A). Co-immunoprecipitation of EGF receptor and integrins was corroborated further by using cell surface biotinylated ECV304 cells kept in suspension or plated on dishes coated with collagen. A 170 kDa band corresponding to the EGF receptor was found in the β1 immunoprecipitate from cells adherent to collagen, but not from cells kept

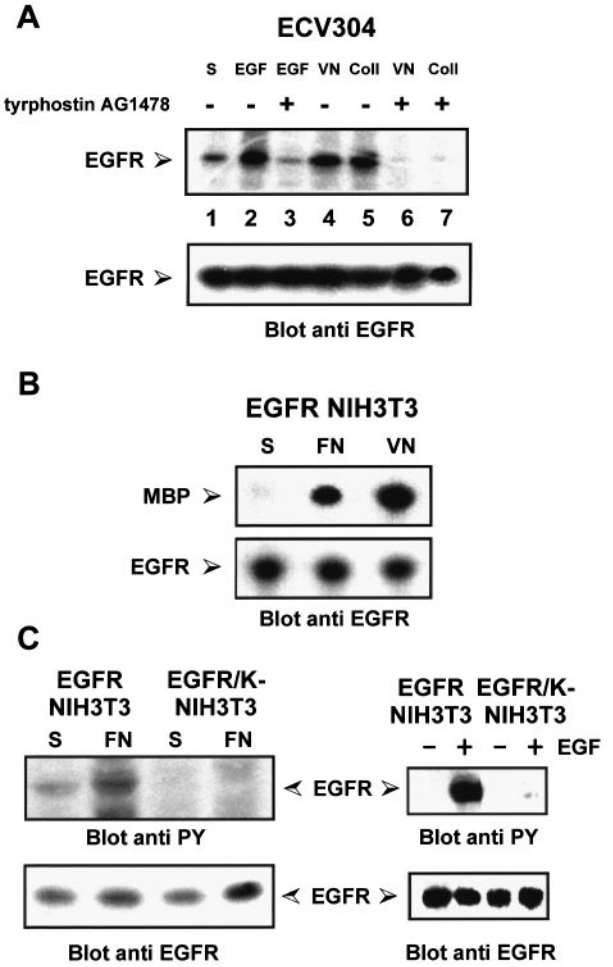

Fig. 4. Analysis of EGF receptor autophosphorylation. (**A**) A kinase assay was performed on EGF receptor immunoprecipitated from ECV304 endothelial cells kept in suspension (S) (lane 1) or allowed to adhere on either 10 μg/ml vitronectin (VN) (lanes 4 and 6) or collagen type I (Coll) (lanes 5 and 7) for 30 min in the absence (lanes 1, 4 and 5) or presence of 250 nM tyrphostin AG1478 (lanes 6 and 7). Confluent cultures were treated with 15 ng/ml EGF (lanes 2 and 3) in the absence (lane 2) or presence (lane 3) of 250 nM tyrphostin AG1478. (**B**) A kinase assay was performed on EGF receptor immunoprecipitated from ECV304 endothelial cells kept in suspension (S) or allowed to adhere on either 10 μg/ml FN or VN for 30 min in the presence of MBP as endogenous substrate. (**C**) Upper left panel: EGFR NIH-3T3 or EGFR kinase-negative (EGFR/K$^-$) NIH-3T3 cells were kept in suspension (S) or allowed to adhere on 10 μg/ml FN for 30 min. Cell extracts were resolved by SDS–PAGE and immunoblotted with anti-PY mAb PY20. Upper right panel: cell extracts of EGFR NIH-3T3 or EGFR kinase-negative (EGFR/K$^-$) NIH-3T3 cells stimulated with 15 ng/ml EGF (+) or left unstimulated (–) were immunoprecipitated with the EGF receptor polyclonal antibody 2913, run on SDS–PAGE and immunoblotted with anti-PY mAb PY20 (upper panel). Filters were reprobed with polyclonal antibody EGFR1 (lower panels).

in suspension (Figure 3B, lanes 1 and 2). Similarly, a band corresponding to the β1 integrin was detectable in the anti-EGF receptor immunoprecipitate (Figure 3B, lanes 3 and 4), suggesting that integrins and EGF receptor can form a complex on the cell membrane. Densitometric analysis showed that the fraction of EGF receptor associated with the β1 integrin represents approximately one-tenth of the total biotinylated cell surface EGF receptor, suggesting that the integrin-associated receptor might represent a subset of the receptor molecules.

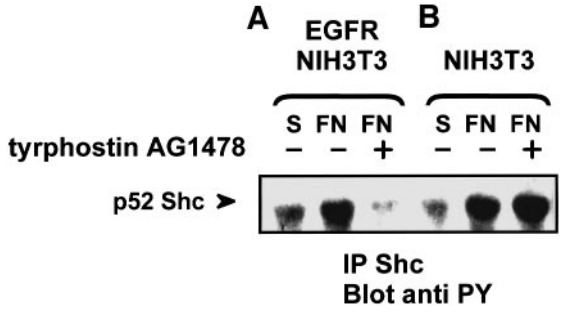

Fig. 5. Analysis of adhesion-induced Shc tyrosine phosphorylation. EGFR NIH-3T3 (**A**) and parental NIH-3T3 cells (**B**) were kept in suspension (S) or allowed to adhere on 10 μg/ml fibronectin (FN) for 30 min in the absence (–) or presence (+) of 250 nM tyrphostin AG1478. Shc protein was immunoprecipitated from cell extracts using a specific polyclonal antiserum and subsequently immunoblotted with anti-PY mAb RC20. The p52 Shc isoform is indicated by the arrow.

Integrin-mediated adhesion triggers autophosphorylation of EGF receptor

To investigate whether integrin-mediated adhesion stimulates the intrinsic receptor kinase activity, we performed kinase assays on EGF receptor immunoprecipitated from extracts of cells either plated on matrix proteins or kept in suspension. As shown in Figure 4A, EGF receptor kinase activity is 2-fold higher in cells attached to matrix than in cells in suspension (lanes 1, 4 and 5). Similar results were obtained by analyzing EGF receptor kinase activity using myelin basic protein (MBP) as exogenous substrate (Figure 4B) (Wang *et al*., 1991). Activation of EGF receptor kinase induced by matrix proteins was lower than that induced with 15 ng/ml EGF (Figure 4A, lane 2). To test if adhesion induces the intrinsic receptor kinase activity, we used tyrphostin AG1478, a very potent and specific inhibitor of EGF receptor kinase (Levitzki and Gazit, 1995). Tyrphostin AG1478 abolished the EGF receptor kinase activity both in matrix-adherent and in EGF-treated cells (Figure 4A, lanes 3, 6 and 7). In addition, we tested NIH-3T3 cells expressing the kinase-negative EGF receptor mutant (EGFR/K$^-$ NIH-3T3) (Figure 4C, right panel) (Sorkin *et al*., 1992). When these cells were plated on fibronectin, neither tyrosine phosphorylation of the receptor (Figure 4C, left panel) nor kinase activity (data not shown) were detected. Therefore, we can conclude that integrin-mediated tyrosine phosphorylation of the EGF receptor requires activation of receptor kinase activity.

Adhesion-induced EGF receptor phosphorylation leads to Erk-1/MAP kinase activation

The adaptor Shc is a well-known downstream effector of the EGF receptor pathway (Pelicci *et al*., 1992), leading to Erk-1/MAP kinase activation. We tested whether integrin-dependent EGF receptor phosphorylation activates this pathway. Immunoprecipitation experiments indicated that the p52 Shc isoform was tyrosine phosphorylated following adhesion of EGFR-transfected NIH-3T3 cells to fibronectin. Adhesion-induced Shc tyrosine phosphorylation was abolished by treatment with tyrphostin AG1478, indicating that EGF receptor tyrosine kinase is required for this event (Figure 5A). Co-precipitation of EGF receptor and Shc in cells adherent to matrix proteins (data not shown) further confirms that integrin-mediated EGF receptor activation leads to association of Shc. In contrast,

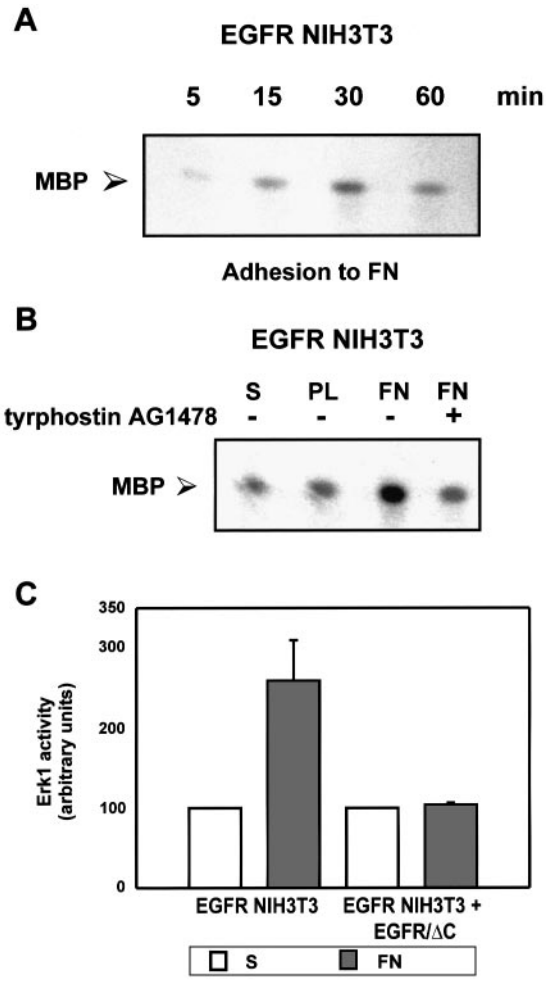

Fig. 6. Involvement of EGF receptor phosphorylation in integrin-mediated Erk-1 MAP kinase activation. Erk-1 MAP kinase activity was determined using MBP as exogenous substrate as described in Materials and methods, and phosphorylated MBP (arrow) was visualized by autoradiography after gel electrophoresis (A and B). (**A**) EGFR NIH-3T3 cells were plated on 10 μg/ml fibronectin (FN) for the indicated times. Note a peak of Erk-1 activity at 30 min of adhesion. (**B**) EGFR NIH-3T3 cells were kept in suspension (S) or plated either on 10 μg/ml poly-L-lysine (PL) or FN for 30 min in the presence (+) or absence (–) of 250 nM tyrphostin AG1478. (**C**) EGFR NIH-3T3 and EGFR NIH-3T3 cells co-transfected with the dominant-negative form of the EGF receptor (EGFR NIH3T3 + EGFR/ΔC) were kept in suspension (S, white bars) or plated on 10 μg/ml fibronectin (FN, gray bars). Phosphorylated MBP was quantified by densitometric analysis. Data are shown as the mean ± SE of five independent experiments.

in parental NIH-3T3 cells, which express very low levels of endogenous EGF receptors (~5×10^3 molecules per cell) (Sorkin *et al*., 1992), p52 Shc was phosphorylated following adhesion, but this phosphorylation was not reduced by tyrphostin AG1478 treatment (Figure 5B), suggesting that in these cells, Shc phosphorylation following adhesion does not depend on EGF receptor activation.

To assess activation of the Erk-1/MAP kinase pathway, EGFR-transfected NIH-3T3 cells were plated on fibronectin for different times, and Erk-1/MAP kinase activity was evaluated. Erk-1 activity was maximal after 30 min of adhesion to fibronectin, and decreased after 1 h (Figure 6A). Comparison between the time course of adhesion-induced Erk-1 activation and EGF receptor tyrosine phosphorylation indicates that the two processes reach maximal levels with similar kinetics (data not shown). If cells were treated with tyrphostin AG1478 specifically to inhibit EGF receptor kinase, Erk-1 activity in response to adhesion was almost completely inhibited (Figure 6B). Similarly, co-expression of a dominant-negative receptor mutant, lacking almost the entire cytoplasmic domain, EGFR/ΔC, caused a dramatic inhibition of Erk-1 activation by adhesion (Figure 6C). Thus adhesion-dependent Erk-1/MAP kinase induction requires EGF receptor activation. To confirm these results in cells naturally expressing EGF receptor, we tested Erk-1 activation in ECV304 cells by clustering β1, β3 or αv subunits by specific antibodies. Quantitative analysis showed that in these cells Erk-1 activation was reduced by tyrphostin AG1478 treatment (Figure 7A). Interestingly, in parental NIH-3T3 cells, Erk-1 was activated by adhesion, but this activation was not reduced by tyrphostin AG1478 treatment (Figure 7B). Thus, the use of both tyrphostin and a dominant-negative form of the receptor indicates that EGF receptor activation by adhesion largely contributes to the integrin signaling towards activation of the Erk-1/MAP kinase pathway in cells such as primary fibroblasts, ECV or NIH-3T3 cells transfected with the EGF receptor. All these cells express $>3\times10^4$ EGF receptors per cell; when EGF receptor is barely expressed, such as in parental NIH-3T3 cells ($<5\times10^3$ molecules per cell), however, Erk-1/MAP kinase activation in response to integrins is controlled predominantly by an EGF receptor-independent alternative pathway.

Since EGF is known to activate MAP kinases through its receptor, we tested whether integrin-induced EGF receptor-dependent Erk-1/MAP kinase activity can still be modulated by EGF addition in adherent cells. Fibronectin-dependent Erk-1 activation was increased 2-fold when 15 ng/ml EGF were added to EGFR-transfected NIH-3T3 cells, indicating that integrin-mediated pathways are additive to that of the natural ligand (Figure 7C).

Adhesion-induced EGF receptor activation does not induce cell proliferation, but protects cells from apoptosis

To investigate whether integrin-mediated EGF receptor activation may influence the ability of cells to progress into the cell cycle, serum-deprived EGFR-transfected NIH-3T3 cells were plated on fibronectin in the absence and presence of EGF or serum. Entry into the cell cycle was measured as the percentage of cells in G_2/M phase 24 h after plating. As shown in Table I, cells plated on fibronectin do not progress into G_2/M phase unless EGF or serum is also present. Thus, transient EGF receptor and Erk-1/MAP kinase activation in response to fibronectin does not lead to a loss of requirement for growth factors to progress into the cell cycle. Adhesion, however, is important for entry into the cell cycle, since EGF or serum do not trigger entry into G_2/M phases in cells kept in suspension (data not shown).

Many cell types undergo apoptosis when deprived of adhesion to the appropriate extracellular matrix. As shown in Figure 8, EGFR-transfected NIH-3T3 cells kept in suspension for 24 h in serum-free medium enter apoptosis, while their plating on fibronectin in the absence of growth factors protects them from programmed cell death. Tyrphostin AG1478 reduced survival, and the dominant-

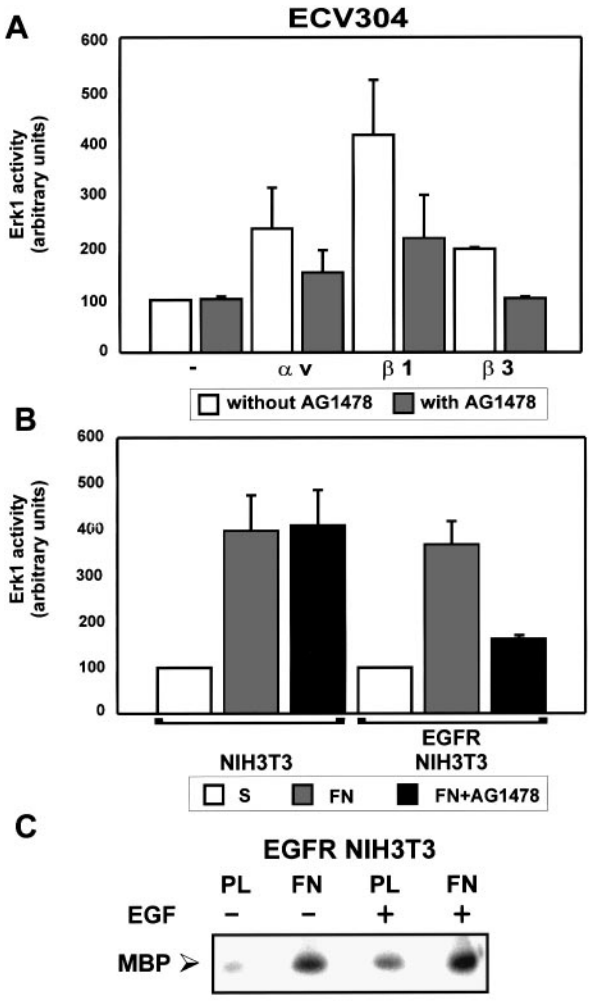

Fig. 7. Analysis of integrin-dependent Erk-1 MAP kinase activation. Immunoprecipitated Erk-1 was subjected to kinase assay and phosphorylated MBP was visualized by autoradiography after gel electrophoresis. Phosphorylated MBP was quantified by densitometric analysis. Data are shown as the mean ± SE of four independent experiments. (**A**) ECV304 cells kept in suspension were incubated for 30 min at 4°C in serum-free DMEM (–) containing 10 μg/ml mAbs L230 to αv, BV7 to β1, or B212 to β3 integrin subunits. Integrin clustering was induced by incubating cells with 20 μg/ml rabbit anti-mouse IgG for 30 min at 37°C, in the absence (white bars) or presence (gray bars) of 250 nM tyrphostin AG1478. (**B**) Parental NIH-3T3 or EGFR NIH-3T3 cells were kept in suspension (S, white bars) or seeded on 10 μg/ml fibronectin (FN) in the absence (gray bars) or presence (black bars) of 250 nM tyrphostin AG1478 for 30 min. (**C**) EGFR NIH-3T3 cells were plated on 10 μg/ml poly-L-lysine (PL) or fibronectin (FN) for 30 min, in the absence (–) or presence (+) of 15 ng/ml EGF.

negative EGF receptor mutant, EGFR/ΔC, completely abolished the protection induced by fibronectin. Moreover, tyrphostin AG1478 partially abolished the fibronectin-mediated survival effect in ECV304 cells, indicating that the EGF receptor is physiologically required in the cell survival response triggered by the extracellular matrix. Thus, adhesion-induced EGF receptor activation is an essential step in cell survival exerted by the extracellular matrix. To confirm further the involvement of EGF receptor in the survival pathway, we also found that parental NIH-3T3 cells are not sensitive to tyrphostin treatment, suggesting that in these cells fibronectin-mediated cell survival is regulated by pathways distinct from the activation of EGF receptor (Figure 8). The addition of the MAP kinase kinase MEK inhibitor, PD98059 (Dudley *et al*., 1995), does not abolish the ability of EGFR-transfected NIH-3T3 cells to survive on fibronectin, indicating that the Erk-1/MAP kinase is not required for this response. In contrast, a strong inhibition of the survival of EGFR-transfected NIH-3T3 cells on fibronectin was induced by treating cells with the phosphatidylinositol-3 kinase (PtdIns-3 kinase) inhibitor wortmannin (Figure 8), while parental NIH-3T3 cells were unaffected by wortmannin treatment (not shown). Therefore, these data show that adhesion-induced EGF receptor signaling is involved in fibronectin-mediated cell survival through a pathway distinct from Erk-1/MAP kinase activation, which may involve PtdIns-3 kinase activation.

Table I. Analysis of cell cycle progression

Percentage of cells in the G_2/M phase of the cell cycle				
Culture	FN	FN + EGF	FN + FCS	S + FCS
17.98 ± 7.03	0.00 ± 0.00	4.43 ± 0.08	11.14 ± 2.55	0.00 ± 0.00

EGFR NIH-3T3 cells were serum deprived for 30 h, detached and kept in suspension (S) or plated on 10 μg/ml fibronectin (FN) for 24 h in the presence of 50 ng/ml human recombinant EGF or 10% FCS where indicated. Cells in culture were used as positive control. Cells were stained with propidium iodide, and the percentage of cells in G_2/M phase was determined by flow cytometry according to the Bio-Rad DNA-Analysis software. Data are shown as the mean ± SE of three representative independent experiments.

Discussion

In this study, we demonstrate that in human primary fibroblasts, endothelial cells and EGFR-transfected NIH-3T3 cells, adhesion to extracellular matrix proteins induces a transient tyrosine phosphorylation of the EGF receptor, which in turn leads to Erk-1/MAP kinase activation and to anchorage-dependent cell survival. Activation of the EGF receptor by cell–matrix adhesion is a new pathway linking integrin signaling to nuclear events.

The data reported in this work provide new evidence on the ability of integrins to co-operate directly at the membrane level with growth factor receptors in order to activate specific signaling pathways. Integrin-dependent tyrosine phosphorylation of the EGF receptor is inhibited by tyrphostin AG1478, a drug which specifically inhibits EGF receptor kinase competing for ATP (Levitzki and Gazit, 1995). The use of a kinase-negative EGF receptor confirms that tyrosine phosphorylation of EGF receptor by integrins is due to *bona fide* activation of intrinsic receptor kinase activity. This event is unlikely to depend on autocrine or paracrine production of EGF receptor ligands during cell adhesion. In fact, it was shown previously that NIH-3T3 cells transfected with the EGF receptor do not produce EGF receptor ligands capable of autocrine activity (Di Fiore *et al*., 1987; Velu *et al*., 1987). In addition, primary fibroblasts or ECV304 cells do not express EGF or TGF-α transcripts, and their conditioned medium is unable to stimulate EGF receptor tyrosine phosphorylation, indicating that other EGF-like ligands such as β-cellulin, heparin-binding EGF or amphiregulin

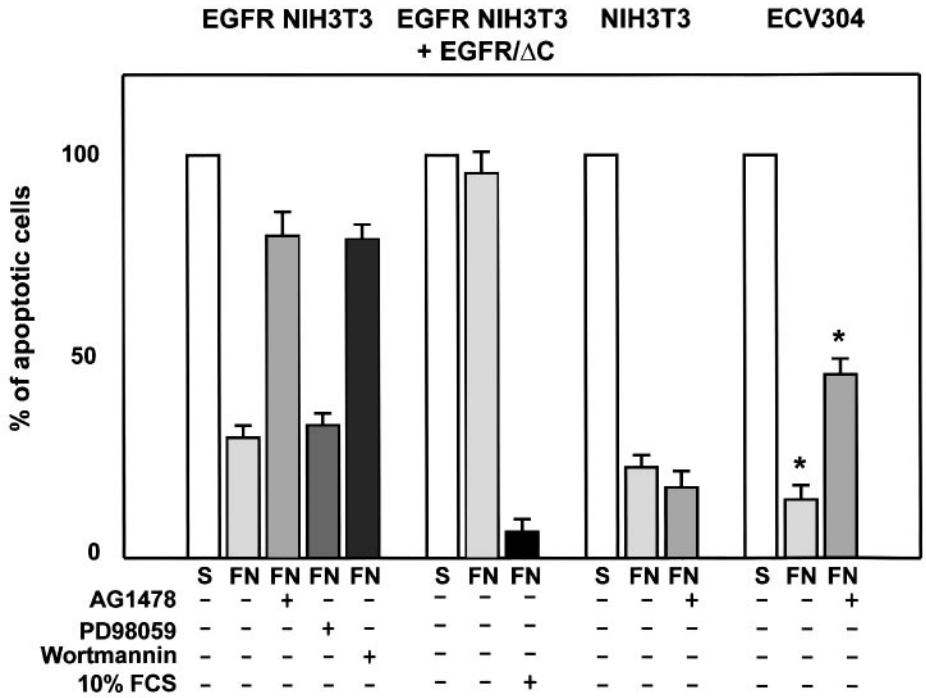

Fig. 8. Involvement of EGF receptor activation in adhesion-mediated cell survival to apoptosis. EGFR NIH-3T3, EGFR NIH-3T3 co-transfected with the dominant-negative form of the EGF receptor (EGFR NIH3T3 + EGFR/ΔC), parental NIH-3T3 and ECV304 cells were kept in suspension on polyHEMA-coated dishes (S), or allowed to adhere on 10 μg/ml fibronectin (FN) for 24 h in the absence (–) or presence (+) of 250 nM tyrphostin AG1478, 25 μM PD98059, 200 nM wortmannin or 10% FCS. Cells were stained with propidium iodide and examined by laser-scanning confocal microscope. The data were reported as the percentage of apoptotic cells over 400 nuclei scored for each sample. Data are shown as the mean ± SE of five independent experiments. *Differences at $P = 0.0001$.

(reviewed in Pinkas-Kramarski *et al*., 1997) do not contribute to receptor activation in our cellular systems.

The data reported in this work, moreover, indicate that integrin-mediated activation of the EGF receptor is not limited to its tyrosine phosphorylation, but downstream signaling events are also generated. In particular, we have shown that integrin-induced EGF receptor phosphorylation leads to a well-defined EGF receptor pathway, consisting of Shc phosphorylation and activation of Erk-1/MAP kinase. Other mechanisms of MAP kinase activation by integrins have been reported. In particular, a subset of integrin heterodimers can associate with caveolin and promote Shc tyrosine phosphorylation, leading to Ras–MAP kinase activation (Wary *et al*., 1996). Moreover, in cells overexpressing p125Fak, this phosphorylated molecule can recruit Grb2 and lead to activation of Ras–MAP kinase (Schlaepfer and Hunter, 1997). The activation of MAP kinase by the EGF receptor thus represents a novel mechanism by which integrins can activate this pathway. The ability of tyrphostin AG1478 and of the dominant-negative EGF receptor mutant (EGFR/ΔC) to strongly decrease or abolish this integrin-dependent signaling indicates that in our cellular system EGF receptor is a major transducing element in this pathway. Interestingly, tyrphostin AG1478 did not affect integrin-induced MAP kinase activation in parental NIH-3T3 cells (see Figure 6), indicating that an EGF receptor-independent pathway, most likely involving Shc–caveolin complexes as described by Wary *et al*. (1996), is utilized in these cells to regulate MAP kinase activation in response to extracellular matrix. Since parental NIH-3T3 cells express very low levels of EGF receptors ($\sim 5\times10^3$) compared with endothelial cells, human fibroblasts and EGFR-transfected NIH-3T3 cells ($>3\times10^4$ molecules per cell), we propose that integrins utilize the EGF receptor as a transducing molecule to activate MAP kinases when this receptor is expressed above a threshold level.

The level of EGF receptor phosphorylation induced by integrins can be increased strongly by addition of EGF (see Figure 1D), indicating that integrins induce only partial activation of the EGF receptor. The partial activation of the receptor is also confirmed by the fact that Erk-1/MAP kinase activation induced by integrin-mediated adhesion is lower than that obtained in response to EGF in adherent cells. The level of Erk-1/MAP kinase activation induced by the integrin-dependent EGF receptor pathway is not sufficient to promote cell growth, consistent with the finding that G_1-arrested EGFR-transfected NIH-3T3 cells do not progress into the cell cycle when plated on fibronectin in the absence of growth factors. Proliferation was observed in cells exposed to fibronectin in the presence of EGF, suggesting that the increased level of MAP kinase activation obtained by co-stimulation with matrix proteins and EGF is necessary to induce cell proliferation (see Table I). The requirement for signaling originating from cell adhesion and from growth factor stimulation to induce MAP kinase activation has also been reported by other investigators (Zhu and Assoian, 1995; Lin *et al*., 1997; Renshaw *et al*., 1997). Therefore, we propose a model in which a threshold level of growth factor receptor activation induced by cell–matrix interaction is required in order to obtain the full response of the receptor to its ligand. Other investigators have shown that EGF stimulates EGF receptor tyrosine phosphorylation and MAP kinase induc-

tion more efficiently when integrins are occupied (Cybulsky *et al*., 1994; Miyamoto *et al*., 1996; Cybulsky and McTavish, 1997; Jones *et al*., 1997). These authors did not detect in their systems the integrin-dependent EGF receptor activation which we report here, most probably due to the different experimental protocols used. In fact, their cells were always maintained in the presence of EGF or serum, a possible source of TGF-α. The ability of integrins to transactivate EGF receptor, as reported in our work, can thus represent a molecular mechanism at the basis of this phenomenon. Assuming that integrins can transactivate growth factor receptors other than the EGF receptor, the data presented here can also explain the ability of integrins to potentiate signaling pathways in response to insulin and PDGF, as shown by Vuori and Ruoslahti (1994) and Schneller *et al*. (1997). These authors reported that upon growth factor stimulation, both insulin and PDGF-β receptors are highly tyrosine phosphorylated and bind to several signaling molecules, such as IRS-1, PLCγ, Ras GAP, the p85 subunit of PtdIns-3 kinase and the tyrosine phosphatase SHP2, when αvβ3 integrin is occupied by its matrix ligand. In endothelial cells, moreover, we have shown that αvβ3 integrin can potentiate the activation of vascular endothelial growth factor receptor by its ligand (R.Soldi, S.Mitola, M.Strasly, P.Defilippi, G.Tarone and F.Bussolino, submitted). Indeed, adhesion-dependent growth factor receptor activation is not restricted to the EGF receptor. It has been shown, in fact, that cell–substratum interactions stimulate phosphorylation of the hepatocyte growth factor (HGF) receptor (Rusciano *et al*., 1996; Wang *et al*., 1996) and PDGFβ receptor (Sundberg and Rubin, 1996). Moreover, cell–cell interactions mediated by the neural cell adhesion molecule activate the fibroblast growth factor receptor in neuronal cells (Saffell *et al*., 1997), suggesting that activation of growth factor receptors in the absence of their specific ligands can be a broadly used mechanism in adhesion-mediated signaling and represents a priming step in order to obtain a full response to growth factor.

The molecular mechanisms underlying EGF receptor activation by integrins remain to be defined. Our findings indicate that integrin-dependent activation of the EGF receptor and of other tyrosine kinases such as p125Fak occurs through distinct mechanisms. In fact, cytochalasin D, which disrupts the actin cytoskeleton, strongly inhibits p125Fak tyrosine phosphorylation, but does not affect EGF receptor tyrosine phosphorylation in response to integrins. This indicates that the organization of actin cytoskeleton is not a primary event in integrin-dependent EGF receptor activation, while it is required to bring together signaling proteins leading to p125Fak tyrosine phosphorylation. These data also indicate that p125Fak tyrosine phosphorylation is not required in integrin-mediated EGF receptor activation. Moreover, integrins and EGF receptors can associate on the membrane, forming a molecular complex, as shown by co-immunoprecipitation experiments, while association of integrins with p125Fak cannot be detected by this technique. Preliminary experiments also show that PtdIns-3 kinase is not involved in integrin-induced EGF receptor tyrosine phosphorylation, as detected by the use of specific inhibitors (M.Venturino, L.Dolce and P.Defilippi, unpublished results).

In addition to inducing Erk-1/Map kinase activity, integrin-mediated EGF receptor activation can also generate downstream signaling leading to anchorage-dependent cell survival. It is well known that cells undergo apoptosis when they lose contact with the extracellular matrix (reviewed in Frisch and Ruoslahti, 1997). Integrin-induced tyrosine phosphorylation may indeed represent one signaling mechanism to regulate cell survival in response to adhesion. Indeed, inhibition of tyrosine phosphatases prevents apoptosis in cells in suspension (Meredith *et al*., 1993). Microinjection of p125Fak antibodies (Hungerford *et al*., 1996) induces apoptosis in embryo fibroblasts, while expression of a constitutive active form of this kinase rescues epithelial cells from apoptosis (Frisch *et al*., 1996), indicating that p125Fak is involved in survival signaling. The data reported here show that in cells expressing EGF receptor, treatment with tyrphostin AG1478 strongly reduces survival of cells plated on matrix in the absence of growth factors. Similar results are observed in cells expressing the dominant-negative form of the EGF receptor. The downstream pathway involved is likely to be independent of Erk-1/MAP kinase activation, since the specific inhibitor of the MAP kinase kinase MEK, PD 98059 (Dudley *et al*., 1995), does not interfere with adhesion-induced cell survival. PtdIns-3 kinase has been implicated recently as a key mediator of matrix-induced survival of normal epithelial cells (Khwaja *et al*., 1997). Interestingly, in cells expressing EGF receptor, fibronectin-mediated cell survival is blocked by the PtdIns-3 kinase inhibitor wortmannin, suggesting that PtdIns-3 kinase may be involved in the downstream pathways leading to cell survival. Therefore, integrin-mediated EGF receptor activation constitutes a novel signaling pathway utilized by integrins to promote adhesion-dependent cell survival.

Here we show that the EGF receptor can be activated by interaction with extracellular matrix receptors. The EGF receptor recently has been shown to provide a link to MAPK activation in response to G protein-coupled receptor agonists (Daub *et al*., 1996, 1997; Luttrell *et al*., 1997) and to growth hormone receptor, which belongs to the cytokine receptor superfamily (Yamauchi *et al*., 1997), and to modulate ion channel activity following m1 muscarinic acetylcholine receptor activation (Tsai *et al*., 1997). In addition, a variety of stimuli, including UV irradiation or calcium-dependent responses, result in ligand-independent EGF receptor transactivation (Huang *et al*., 1996; Rosen and Greenberg, 1996), suggesting that the EGF receptor may represent a switch point for multiple stimuli regulating a variety of fundamental physiological processes.

All together, our results demonstrate that the EGF receptor can act as a downstream effector in integrin signaling upon cell–matrix interactions. EGF receptor activation by integrins is partial, compared with that obtained with EGF. Nevertheless, this activation is sufficient to prevent apoptosis induced by cell–matrix detachment, and this event may represent a prerequisite for a full mitogenic response to growth factor stimulation in adherent compared with non-adherent cells. In conclusion, the ability of integrins to activate the EGF receptor and downstream signaling further supports the concept that integrins and growth factor receptors can cooperate directly in the plane of the membrane to trigger specific signaling.

Materials and methods

Reagents and antibodies

Fibronectin and vitronectin were purified from human plasma as previously described (Defilippi *et al*., 1994). Collagen type I, poly-L-lysine, cycloheximide, cytochalasin D, myeline basic protein (MBP), bovine pancreatic RNase, propidium iodide, puromicin, wortmannin, sulfosuccinimidyl biotin and human recombinant EGF were all from Sigma Chemical Co. Tyrphostin AG1478 and PD98059 were from Calbiochem. Protein A–Sepharose was from Pharmacia. [γ-^{32}P]ATP, nitrocellulose, streptavidin–horseradish peroxidase (HRP) conjugate, the ECL reagents and films were from Amersham. Culture media, sera and the Lipofectamine reagent were from Gibco-BRL.

The following antibodies to integrin subunits were used: monoclonal antibody (mAb) BV7 to the human β1 integrin subunit (purchased from Bioline Diagnostici), mAb L230 to the αv integrin subunit purchased from ATTC, mAb B212 to the β3 subunit and the polyclonal antibody to the β1 integrin cytoplasmic domain previously described (Defilippi *et al*., 1991). mAb RI.30 to human β_2-microglobulin was a kind gift of Professor F.Malavasi (University of Ancona, Italy). All the monoclonal antibodies were affinity purified on protein A–Sepharose as described (Ey *et al*., 1978), and the purity of the antibodies was >95%. Antibodies to the EGF receptor were: mAb HB-8505 (purchased from ATCC), polyclonal antibody 2913 (Beguinot *et al*., 1986) and polyclonal antibody EGFR1, produced by injection of a 13 amino acid peptide corresponding to the C-terminus of the human EGF receptor (NH_2-LRVAPQSSEFIGA-COOH). Polyclonal antibody to p125Fak Fak4 has been described previously (Defilippi *et al*., 1995). Polyclonal antibody to Shc was produced by injection of a GST fusion protein corresponding to amino acid residues 1180–1620 of human Shc (Pellicci *et al*., 1992). Rabbit anti-mouse IgG was produced and purified in our laboratory. mAb PY20 and RC20 to phosphotyrosine (anti-PY) were from Transduction Laboratories. Polyclonal antibody C-16 to Erk-1/MAP kinase was from Santa Cruz Biotechnology.

Cell culture and transfection

Human dermal fibroblasts were prepared from skin biopses of a healthy donor and stabilized in culture according to established procedures. Human endothelial cell line ECV304 was a kind gift of Dr R.Pardi (Dibit, Milano). NIH-3T3 cells tranfected with wild-type human EGF receptor (EGFR NIH-3T3) (Velu *et al*., 1989) or EGF receptor kinase-negative point mutants (EGFR/K$^-$) have been described previously (Sorkin *et al*., 1992; Alvarez *et al*., 1995). The dominant-negative EGF receptor mutant lacking all the kinase and C-terminal domains (EGFR/ΔC) was prepared by PCR mutagenesis by inserting point mutations in EGF receptor cDNA in positions 2248 and 2257, converting Glu633 and Glu666 into stop codons. PCRs were performed in the pMMTV-EGFR as previously described (Sorkin *et al*., 1996) using an upstream primer 5′-TACGCAGACGCCGGCCATGTGTGC-3′ (2014–2036, *Nae*I) and downstream primer 5′-GACCATGATCATGTA-GACATCGATGGGT-3′ (3033–2994, *Cla*I). For mutagenesis, primers 5′-CTG-CAGGAGAGG**T**AGCTTGTG**T**AGCCTTACA-3′ (226–2268, direct) and 5′-TGTAAGGAGGCT**A**CACAAG**A**TCCCTCCCTGCAG-3′ (2268–2236, inverse) were used. The *Nae*I–*Cla*I-mutated fragment was recloned back into the pMMTV-EGFR and sequenced to confirm the mutations and ensure the absence of secondary mutation. The *Not*I–*Eco*RV fragment of EGFR/ΔC cDNA was subcloned into the pRK-5 vector (pRK-EGFR/ΔC). NIH-3T3 cells expressing wild-type EGF receptor were transfected by the Lipofectamine method with pRK-EGFR/ΔC together with the pGKpuro plasmid encoding the puromycin-resistant gene, and cells were selected with 3.5 μg/ml puromicin. To analyze expression of the EGFR/ΔC as a protein migrating at 130 kDa, cells were cell surface biotinylated as described previously (Defilippi *et al*., 1997b), and EGF receptor was immunoprecipitated with mAb HB-8509 and revealed with streptavidin–HRP (Amersham).

Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) (N6 or ECV304) or newborn calf serum (NIH-3T3 cells).

Adhesion assay and integrin clustering

Cells grown to confluence were serum deprived in DMEM for 16–24 h, pre-treated for 2 h with 20 μM cycloheximide, detached with 10 mM EDTA in phosphate-buffered saline (PBS), washed and kept in suspension or plated for the indicated times on poly-L-lysine, matrix proteins or integrin antibody-coated dishes as previously described (Defilippi *et al*., 1994). When indicated, human recombinant EGF, tyrphostin AG1478 or cytochalasin D were added. In the co-immunoprecipitation experiments, cell surface biotinylation was performed as previously described (Defilippi *et al*., 1997b). Cells were then washed with a PBS buffer containing 5 mM EDTA, 10 mM NaF, 10 mM $Na_4P_2O_7$, 0.4 mM Na_3VO_4 and detergent extracted in lysis buffer as described below. Integrin clustering was performed as previously described (Defilippi *et al*., 1994).

Cell lysis, immunoprecipitation and immunoblotting

Cells were extracted with 1% NP-40 lysis buffer (1% NP-40, 150 mM NaCl, 50 mM Tris–HCl pH 8, 5 mM EDTA, 10 mM NaF, 10 mM $Na_4P_2O_7$, 0.4 mM Na_3VO_4, 10 μg/ml leupeptin, 4 μg/ml pepstatin and 0.1 U/ml aprotinin). Cell lysates were centrifuged at 13 000 *g* for 10 min, and the supernatants were collected and assayed for protein concentration with the Bio-Rad protein assay method (Bio-Rad). Aliquots of 100 μg of proteins were run on SDS–PAGE under reducing conditions. For immunoprecipitation experiments, 1–3 mg of proteins were immunoprecipitated with the appropriate antibody for 1 h at 4°C as previously described (Defilippi *et al*., 1991) in the presence of 50 μl of protein A–Sepharose beads. Following SDS–PAGE, proteins were transferred to nitrocellulose, reacted with specific antibodies and then detected with HRP-conjugated secondary antibodies and chemiluminescent ECL detection. When appropriate, the nitrocellulose membranes were stripped according to the manufacturers' recommendations and reprobed.

EGF receptor kinase assay

Cells were extracted in buffer containing 15% glycerol, 150 mM NaCl, 50 mM Tris–HCl pH 8, 5 mM EDTA, 10 mM NaF, 10 mM $Na_4P_2O_7$, 1 mM Na_3VO_4, 10 μg/ml leupeptin, 4 μg/ml pepstatin and 0.1 U/ml aprotinin, immunoprecipitated with EGF receptor polyclonal antibodies, and washed and incubated in EGFR kinase buffer [20 mM PIPES pH 7.2, 10 mM $MnCl_2$, 1 mM dithiothreitol (DTT), 100 μM Na_3VO_4, 10 μM ATP] in the presence of 2 μCi of [γ-^{32}P]ATP at 30°C for 3 min. To measure EGF receptor kinase activity on exogenous substrate, 5 μg of MBP for each sample were added to the reaction mixture. Reactions were terminated by addition of 2× Laemmli buffer and subjected to SDS–PAGE. γ-^{32}P-Labeled EGF receptor was detected by autoradiography and quantified by densitometric analysis using the GS 250 Molecular Imager (Bio-Rad). EGF receptor was quantified by Western blotting with polyclonal antibody EGFR1.

Erk-1 MAPK assay

Erk-1 was immunoprecipitated from cell extracts prepared with 1% NP-40 lysis buffer using 2 μg of polyclonal Erk-1 antibody C-16. Immunoprecipitates additionally were washed with MAPK buffer (50 mM Tris–HCl pH 7.5, 10 mM $MgCl_2$) and incubated in the presence of a 5 μg/sample of MBP and 2 μCi of [γ-^{32}P]ATP in the same buffer at 30°C for 20 min. Reactions were stopped by addition of 2× Laemmli buffer and subjected to SDS–PAGE. γ-^{32}P-Labeled MBP was detected by autoradiography and quantified using the GS 250 Molecular Imager (Bio-Rad).

Analysis of cell cycle progression and apoptosis

To monitor progression to G_2/M phases of the cell cycle, EGFR-transfected NIH-3T3 cells were synchronized by 30 h serum deprivation, detached with 0.05% trypsin, 0.02% EDTA and kept in suspension or plated for 24 h on 10 μg/ml fibronectin-coated dishes in the absence or presence of 10% FCS or 50 ng/ml of human recombinant EGF. Cells were then detached with trypsin/EDTA, fixed in 75% ethanol for 2 min at room temperature and centrifuged. The cell pellet was resuspended in PBS containing 1% NP-40, 1 mg/ml bovine pancreatic RNase and 30 μg/ml propidium iodide for 1 h at room temperature in the dark. Propidium iodide fluorescence was analyzed on the flow cytometer Bryte HS (Bio-Rad), and cell cycle analysis was performed according to the Bio-Rad DNA-Analysis software.

To detect apoptosis, cells were kept in suspension on polyHEMA-coated dishes or plated on fibronectin-coated dishes in serum-free medium in the presence of different mediators for 24 h as previously described (Bozzo *et al*., 1997). Cells were then stained with 25 μg/ml propidium iodide and examined in a Bio-Rad 600 confocal microscope using the argon laser (Bio-Rad Microscience Division, Hercules, CA). Chromatin condensation and nuclear fragmentation were used as morphological criteria to quantify apoptotic cells.

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