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# Integrin-induced Epidermal Growth Factor (EGF) Receptor Activation Requires c-Src and p130Cas and Leads to Phosphorylation of Specific EGF Receptor Tyrosines*

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**Integrin-mediated cell adhesion cooperates with growth factor receptors in the control of cell proliferation, cell survival, and cell migration. One mechanism to explain these synergistic effects is the ability of integrins to induce phosphorylation of growth factor receptors, for instance the epidermal growth factor (EGF) receptor. Here we define some aspects of the molecular mechanisms regulating integrin-dependent EGF receptor phosphorylation. We show that in the early phases of cell adhesion integrins associate with EGF receptors on the cell membrane in a macromolecular complex including the adaptor protein p130Cas and the c-Src kinase, the latter being required for adhesion-dependent assembly of the macromolecular complex. We also show that the integrin cytoplasmic tail, c-Src kinase, and the p130Cas adaptor protein are required for phosphorylation of EGF receptor in response to integrin-mediated adhesion. We show that integrins induce phosphorylation of EGF receptor on tyrosine residues 845, 1068, 1086, and 1173, but not on residue 1148, a major site of phosphorylation in response to EGF. In addition we find that integrin-mediated adhesion increases the amount of EGF receptor expressed on the cell surface. Therefore these data indicate that integrin-mediated adhesion induces assembly of a macromolecular complex containing c-Src and p130Cas and leads to phosphorylation of specific EGF receptor tyrosine residues.**

Integrins are cell surface-adhesive receptors formed by $\alpha$ and $\beta$ subunits, which bind to extracellular matrix proteins. Integrin-mediated adhesion stimulates multiple signaling pathways that modulate actin cytoskeleton organization, cell motility, cell growth, and the ability of cells to escape from apoptosis. Integrin-dependent signaling includes $Ca^{2+}$ influx, cytoplasmic alkalinization, potassium channel activation, tyrosine phosphorylation of cytoplasmic proteins, and activation of the mitogen-activated protein (MAP)[1] kinases ERK-1 and ERK-2 (for review, see Refs. 1–5). 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.

Integrins have been shown to interact with transducing molecules to promote intracellular signaling. Potential candidates as transducing elements are tyrosine kinases of the Fak and Src family. The amino-terminal domain of p125Fak (6, 7) binds *in vitro* the cytoplasmic domain of the $\beta_1$ and $\beta_3$ integrin subunits, whereas its carboxyl-terminal part binds the SH2 and SH3 domains of several proteins involved in focal adhesion assembly and signal transduction (for review, see Ref. 8). After activation by most integrins, p125Fak is phosphorylated on tyrosine 397, which becomes a high affinity binding site for the SH2 domain of c-Src (9). The Src kinase then phosphorylates focal adhesion components, such as the cytoskeletal proteins talin, paxillin, the adaptor p130Cas, and the p125Fak itself on the tyrosine 925, leading to signaling functions. It has been shown that phosphorylated p125Fak interacts with the adaptor molecule Grb-2, leading to MAP kinase activation (10) through a B-Raf-dependent pathway (11). In addition to p125Fak, some $\beta_1$ and $\alpha_v$ integrins activate the Src family member Fyn and the adaptor Shc. The assembly of this transduction complex involves caveolin, a transmembrane protein that cooperates with integrins to activate signaling pathways. After cell-matrix adhesion, integrin-caveolin-Fyn complexes associate with tyrosine-phosphorylated Shc, which, in turn, interacts with the Grb2·Sos complex leading to activation of the Ras-MAP kinase cascade (12). Integrins can also associate with proteins belonging to the Tetraspan family (CD9, CD63, and CD81) to modulate intracellular signaling (13).

Integrin-dependent activation of the small GTPase Rac (for

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[1] The abbreviations used are: MAP, mitogen-activated protein; ERK, extracellular signal-regulated kinase; EGF, epidermal growth factor; mAb, monoclonal antibody; PP1, protein phosphatase 1; MEFs, mouse embryonic fibroblasts; PL, poly-L-lysine; MALDI-TOF, matrix-assisted laser desorption-ionization time-of-flight.

review, see Refs. 14 and 15) has also been proposed recently as an additional mechanism to regulate adhesion-dependent events, such as integrin activation of Jun $NH_2$-terminal kinase (16). Integrin regulation of Rac activation can occur through the adaptor molecules p130Cas and Crk (16, 17), likely through the involvement of a Rac-specific guanine nucleotide exchange factor, such as Vav (18).

In addition to these molecules, growth factor receptors are candidates to cooperate with integrins in assembling a transduction machinery. Integrins have been shown to potentiate signaling pathways in response to insulin, platelet-derived growth factor, epidermal growth factor (EGF), fibroblast growth factor, and vascular endothelial growth factor (19–29). In particular, $\alpha_v\beta_3$ integrin has been shown to synergize with different growth factor receptors. $\alpha_v\beta_3$ integrin occupancy by its matrix ligand is required for full tyrosine phosphorylation of insulin and platelet-derived growth factor $\beta$ receptors and their binding to several signaling molecules such as insulin receptor substrate 1, phospholipase C$\gamma$, Ras GAP, the p85 subunit of phosphatidylinositol 3-kinase and the tyrosine phosphatase SHP2 (19, 22). In endothelial cells, moreover, $\alpha_v\beta_3$ integrin potentiates the activation of vascular endothelial growth factor receptor and of p85 phosphatidylinositol 3-kinase by its ligand (28).

Direct phosphorylation of growth factor receptors by integrin-mediated adhesion represents a potential mechanism by which integrins can enhance signaling pathways emanating from growth factor receptors (30–33).

We have shown recently that in cells expressing more than $10^4$ EGF receptors/cell, integrins induce EGF receptor tyrosine phosphorylation in the absence of EGF receptor ligands, leading to Shc phosphorylation and MAP kinase activation (33). In this work we show that integrins, c-Src, p130Cas, and EGF receptor associate in a macromolecular complex on the cell membrane and that integrin-dependent adhesion induces phosphorylation of specific tyrosine residues of EGF receptor, distinct from those obtained by soluble ligand EGF.

## EXPERIMENTAL PROCEDURES

*Reagents and Antibodies*—The following antibodies to integrin subunits were used: monoclonal antibody (mAb) TS2/16 to the human $\beta_1$ integrin subunit (purchased from ATCC), mAb L230 to the $\alpha_v$ integrin subunit (from ATCC), mAb B212 to the $\beta_3$ subunit, and the polyclonal antibody to the $\beta_1$ integrin cytoplasmic domain described previously (34). All the monoclonal antibodies were affinity purified on protein A-Sepharose as described (35), and the purity of the antibodies was higher than 95%. Antibodies to the EGF receptor were: mAb HB-8509 and HB-8508 (purchased from ATCC), mAb to the activated form of EGF receptor (purchased from Transduction Laboratories), and polyclonal Ab EGFR1 produced as described by Moro *et al*. (33). Polyclonal antibodies to phosphorylated tyrosine 1068, 1086, 1148, and 1173 of the EGF receptor were prepared from BIOSOURCE International. The specificity of each antibody has been tested on extracts of EGF-treated NIH3T3 cells expressing EGF receptor mutated on each specific tyrosine (data not shown). Polyclonal antibody to p125Fak Fak4 has been described previously (33, 36). Rabbit anti-mouse IgGs were produced and purified in our laboratory. mAb PY99 to phosphotyrosine, Crk, and p130Cas were obtained from Transduction Laboratories. mAb to c-Src was from Santa Cruz Biotechnology. Ab to phospho-p60Src (Tyr-416) was a gift from Dr. L. Chen (Cell Signaling Technology).

Human recombinant EGF was from Sigma. 4-amino-5-(4-methylphenyl)-7-(t-butyl)pyrazolo(3,4-d)pyrimidine (PP1) and AG1478 were from Calbiochem. Protein A-Sepharose, nitrocellulose, the ECL reagents, and films were from Amersham Biosciences, Inc. Culture media, sera, and LipofectAMINE reagent were from Invitrogen.

*Cell Culture and Transfection*—Human cell line ECV304 was purchased from ATCC. GD25$\beta$1A and GD25$\beta$1TR cells have been described previously (37). Mouse embryonic fibroblasts (MEFs) isolated from murine Fak−/− and Fak+/+ embryos (38) were a kind gift from Dr. D. Ilic. MEFs isolated from murine p130Cas−/− embryos (39) were a kind gift from Dr. T. Nakamoto. Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. ECV304 cells grown to 80% confluence in 100-mm tissue culture dishes were transiently transfected with the pSGT Src K− plasmid encoding a c-Src point mutant kinase negative (gift from Dr. S. Courtneidge) by the LipofectAMINE reagent as described by the manufacturer. 20 h after transfection the medium was changed to Dulbecco's modified Eagle's medium containing 0.5% fetal calf serum, and cells were incubated for 24 h before the adhesion assay.

*Adhesion Assays*—Cells grown to confluence were serum deprived in Dulbecco's modified Eagle's medium for 24 h, detached with 10 mM EDTA in phosphate-buffered saline, washed, and kept in suspension or plated for 30 min on 10 $\mu$g/ml fibronectin or 10 $\mu$g/ml $\alpha_v$ integrin antibody-coated dishes. In some experiments dishes were coated with poly-L-lysine (PL), a nonspecific adhesive substrate, and postcoated with antibodies to the $\alpha_v$ integrin subunit, a double coating that maximizes the rate of cell adhesion (33, 40). When indicated, human recombinant EGF, PP1, or AG1478 was added at the indicated dose. Cells were then washed with phosphate-buffered saline containing 5 mM EDTA, 10 mM NaF, 10 mM $Na_4P_2O_7$, 1 mM $Na_3VO_4$, and detergent extracted in lysis buffer as described below. In the coimmunoprecipitation experiments, at the end of adhesion cells were incubated for 30 min at 4 °C in the presence of 30 $\mu$g/ml monoclonal antibodies to integrin subunits or to EGF receptor specifically to bind and immunoprecipitate cell surface molecules, washed three times, and detergent extracted.

*Cell Lysis, Immunoprecipitation, and Immunoblotting*—Cells were extracted with 1% Nonidet P-40 lysis buffer (1% Nonidet P-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 $\mu$g/ml leupeptin, 4 $\mu$g/ml pepstatin, and 0.1 unit/ml aprotinin). Cell lysates were centrifuged at 13,000 × *g* for 10 min, and the supernatants were collected and assayed for protein concentration using the Bio-Rad protein assay method. Proteins were run on SDS-PAGE under reducing conditions. For immunoprecipitation experiments, proteins were immunoprecipitated with the appropriate antibody for 1 h at 4 °C as described previously (33) in the presence of 50 $\mu$l of protein A-Sepharose beads. In the coimmunoprecipitation experiments, integrins or EGF receptor were immunoprecipitated from the cell surface. At the end of adhesion, intact cells were incubated with anti-$\beta_3$, anti-$\alpha_v$, or anti-EGF receptor mAbs to bind, respectively, $\alpha_v\beta_3$ integrin or EGF receptor exposed on the cell surface and detergent extracted. Protein A-Sepharose beads were then added to 3 mg of protein cell extract to collect immunoprecipitates. After SDS-PAGE, proteins were transferred to nitrocellulose, reacted with specific antibodies, and then detected with peroxidase-conjugated secondary antibodies and chemoluminescent ECL reagent. When appropriate, the nitrocellulose membranes were stripped according to manufacturer's recommendations and reprobed. Densitometric analysis was performed using the GS 250 molecular imager (Bio-Rad).

*In Gel Tryptic Protein Digestion and Mass Spectrometric Analysis*—EGF receptor-containing bands were cut from the gel and destained overnight with a solution of 50 mM ammonium bicarbonate, 40% ethanol. The protein was digested in gel with trypsin (Promega) according to Hellman *et al.* (41) except that the bands had been washed three times with acetonitrile before drying them in a speed vacuum concentrator.

For matrix-assisted laser desorption-ionization time-of-flight (MALDI-TOF) mass spectrometry, aliquots of 0.5 $\mu$l of the peptide mixtures were applied to a target disc and allowed to air dry. Subsequently, 0.5 $\mu$l of matrix solution (1% w/v $\alpha$-cyano-4-hydroxycinnamic acid in 50% acetonitrile, 0.1% trifluoroacetic acid) was applied to the dried sample and again allowed to dry. Spectra were obtained using a Bruker Biflex III MALDI-TOF spectrometer (Bremen, Germany). For interpretation of the protein fragments the MASCOT program available at the Matrixscience web site (www.matrixscience.com) and the PeptideMass program available at Expasy web site (www.expasy.ch/tools/peptide-mass.html) were used.

*Immunoelectron Microscopy*—For immunoelectron microscopy, 10% gelatin-embedded, 2.3 M sucrose-infused blocks of aldehyde-fixed ECV304 cells were frozen in liquid nitrogen. Ultrathin cryosections were obtained with a Reichert-Jung Ultracut E with FC4E cryoattachment and collected on copper-formvar-carbon-coated grids. Single immunogold localization on ultrathin cryosections was performed as described previously (42, 43). In particular, sections were immunostained with anti-human EGF receptor mAb AB-5 (Oncogene Science) followed by a rabbit anti-mouse bridging antibody (DAKO) and 15-nm protein A-gold. Control sections have been incubated without first antibodies. In all control sections no labeling was detected (not shown). Sections were examined with a Zeiss EM 902 electron microscope.

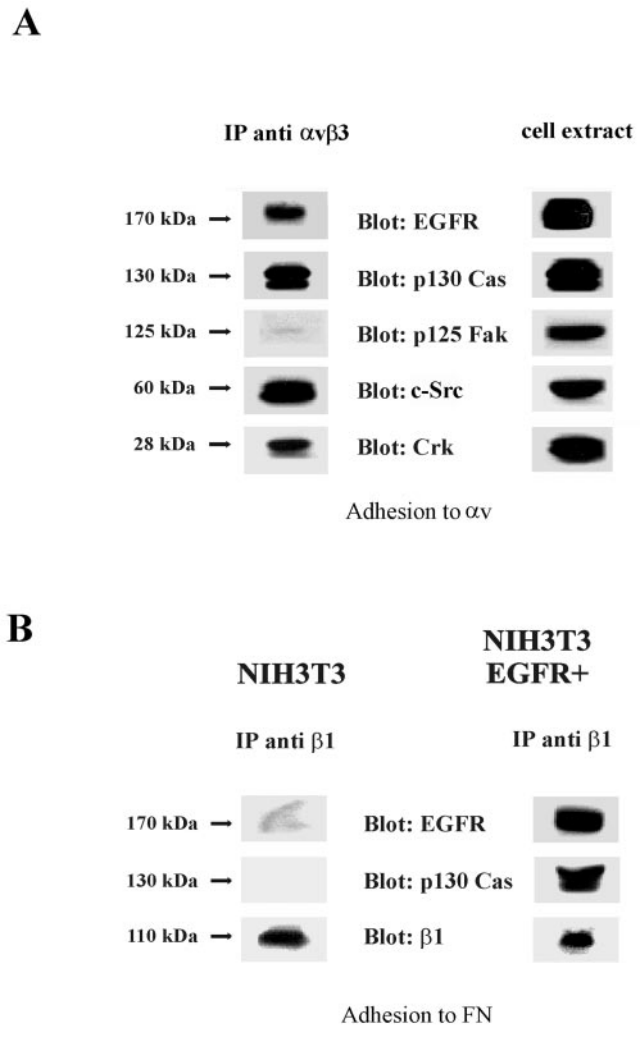



FIG. 1. **Integrins and EGF receptors form a complex in response to adhesion.** *A*, ECV304 cells were detached from culture dishes and plated for 5 min on dishes coated with mAb L230 to the $\alpha_v$ integrin subunit. mAb B212 to the $\beta_3$ subunit was then added to the cells, which were incubated further for 30 min at 4 °C before detergent extraction. Cells extracts were immunoprecipitated by the addition of protein A-Sepharose (*IP anti* $\alpha_v\beta_3$) or run as control on 6% SDS-PAGE and blotted. Immunoblotting was performed with antibodies to EGF receptor (*EGFR*), p130Cas, p125Fak, c-Src, and Crk. *B*, NIH3T3 and NIH3T3 cells transfected with human EGF receptor (EGFR+) were plated on fibronectin-coated dishes for 5 min, and cell extracts were immunoprecipitated with polyclonal antibodies to $\beta_1$ integrin. Materials coimmunoprecipitated with $\beta_1$ integrin were run on gel and blotted, respectively, with the antibodies to EGF receptor, p130Cas, and the $\beta_1$ integrin subunit. The data reported here are representative of 10 distinct experiments.

## RESULTS

*Integrins and EGF Receptor Associate in a Transducing Macromolecular Complex*—We have shown recently that adhesion of human primary skin fibroblasts and ECV304 human cells to immobilized matrix proteins or to antibodies to integrin subunits stimulates tyrosine phosphorylation of the EGF receptor and association with $\beta_1$ integrin (33). To investigate the molecular nature of the complex between integrins and the EGF receptor, we immunoprecipitated $\alpha_v\beta_3$ integrins from ECV304 cells adherent to $\alpha_v$ ligand and performed Western blotting experiments. Fig. 1*A* (*top left panel*) is an example of an integrin-EGF receptor complex identified in ECV304 cells. Western blotting with specific antibodies show that, in addition to the EGF receptor, the adaptor molecules p130Cas, Crk, and c-Src kinase, but not p125Fak, coimmunoprecipitate with $\alpha_v\beta_3$ integrin upon integrin-mediated adhesion (Fig. 1*A*, *left panel*). Similar results were obtained by immunoprecipitating $\beta_1$ integrin in cells plated on fibronectin (Fig. 1*B* and data not shown). Coimmunoprecipitation of the c-Src·Cas·Crk complex with integrins is strictly dependent on the presence of the EGF receptor because these molecules organize in a macromolecular complex only in ECV304 and EGF receptor-transfected NIH3T3 cells, expressing appreciably level of EGF receptor (20-40,000 molecules/cell), but not in wild type NIH3T3 that express barely detectable level of EGF receptor (Fig. 1*B*).

*Integrin-EGF Receptor Macromolecular Complex Is Transiently Assembled*—To investigate the kinetics of macromolecular complex formation, ECV304 cells were plated on dishes coated with $\alpha_v$ ligand, and assembly of the macromolecular complex was analyzed at 5 and 15 min of adhesion. Integrins were immunoprecipitated from the cell surface, and immunoprecipitates were probed with antibodies to the EGF receptor, p130Cas, and c-Src kinase. Western blotting experiments indicate that these distinct components associate only when integrin is engaged and not when cells are attached on PL (Fig. 2*A*) or kept in suspension (not shown). The macromolecular complex is clearly visible within 5 min of adhesion and becomes undetectable at 15 min, indicating that the association between these molecules is an early and transient event. The same kinetics is also obtained by immunoprecipitating the EGF receptor and blotting with antibodies for each component (data not shown). Previous results show that tyrosine phosphorylation of the EGF receptor is maximal within 30 min after plating on matrix proteins and then decreases, reaching basal levels within 4 h (33). In the experiments reported here, when cells were plated on integrin ligands, the EGF receptor was already phosphorylated at 5 min (Fig. 2*B*), showing that complex formation occurs concomitantly to EGF receptor phosphorylation. At 30 min of adhesion, when the complex is disassembled, tyrosine phosphorylation of EGF receptors remains high, indicating that at later times the kinetics of the two events are distinct. Therefore these results indicate that in the early phases of cells adhesion, integrin occupation leads to their association with EGF receptors in a transient macromolecular complex leading to sustained EGF receptor phosphorylation.

*c-Src and EGF Receptor Kinases Are Both Required for Association of Integrin-EGF Receptor Macromolecular Complex*—We analyzed the activation state of the c-Src kinase present in the integrin-EGF receptor macromolecular complex using an antibody that recognizes phosphorylation of the critical tyrosine residue 416 in the Src kinase domain. c-Src is phosphorylated on tyrosine 416 when ECV304 cells are plated on integrin ligands, indicating that integrin-mediated adhesion induces c-Src kinase autophosphorylation (Fig. 3*A*). In ECV304 cells plated on $\alpha_v$ ligand for 5 min, c-Src phosphorylated on tyrosine 416 is detectable in integrin immunoprecipitates from adherent cells but is absent in cells plated on PL (Fig. 3*B*, *bottom panel*), indicating that c-Src is activated after adhesion and that activated c-Src complexes with integrins and EGF receptors. To test whether c-Src kinase activity is required for assembly of the integrin-EGF receptor complex, coimmunoprecipitation experiments were performed in cells exposed to PP1, a specific Src kinase inhibitor (Fig. 3*A*). After PP1 treatment, EGF receptors, p130Cas, and c-Src were not detectable in the immunoprecipitates of $\alpha_v\beta_3$ integrin (Fig. 3*B*), suggesting that inhibition of c-Src kinase activity prevents macromolecular complex assembly. These results were confirmed by expression of a kinase negative form of c-Src. In ECV304 cells expressing the mutant Src kinase form the amount of EGF receptor in the integrin immunoprecipitate is strongly reduced, as well as that of p130Cas and c-Src (Fig. 3*C*). Similar results were obtained in

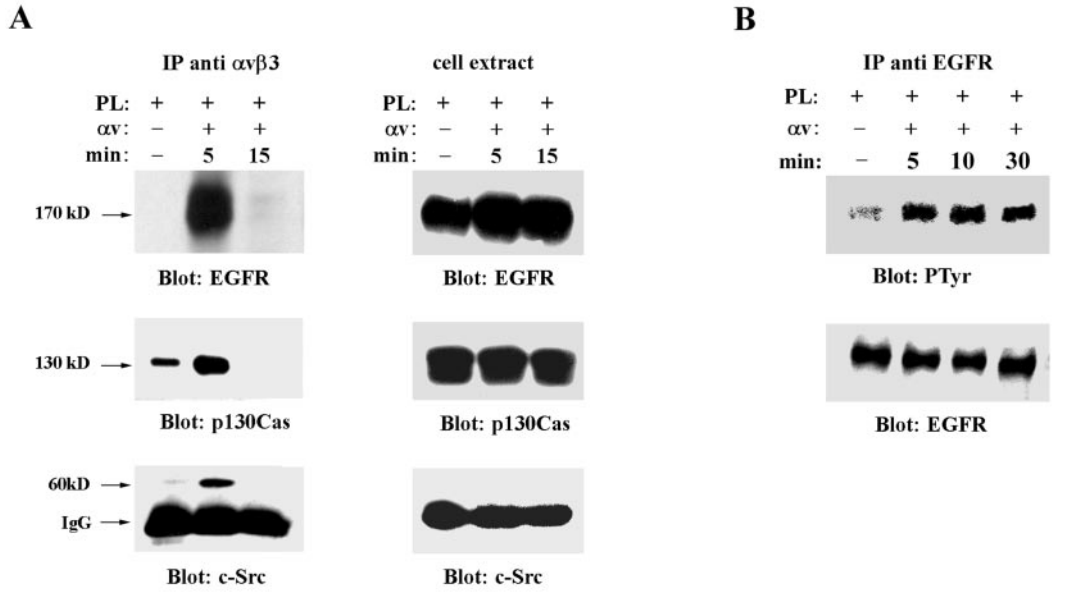



FIG. 2. **Kinetics of macromolecular complex association.** ECV304 cells were plated for different times on dishes coated with PL and postcoated with mAb L230 to the $\alpha_v$ integrin subunit. *A*, mAb B212 to the $\beta_3$ subunit was added to the cells, which were incubated further for 30 min at 4 °C before detergent extraction. Cell extracts were immunoprecipitated by the addition of protein A-Sepharose (*IP anti* $\alpha_v\beta_3$), run on 6% SDS-PAGE, and immunoblotted with antibodies to EGF receptor (*EGFR*), p130Cas, and c-Src, as shown in Fig. 1. *B*, cells were detergent extracted at the indicated times, and extracts were immunoprecipitated by antibodies to EGF receptor. The immunoprecipitates were blotted with antibody PY99 to phosphotyrosine (*upper panel*) and reblotted with polyclonal antibodies to EGF receptor (*lower panel*). The data reported here are representative of four distinct experiments.

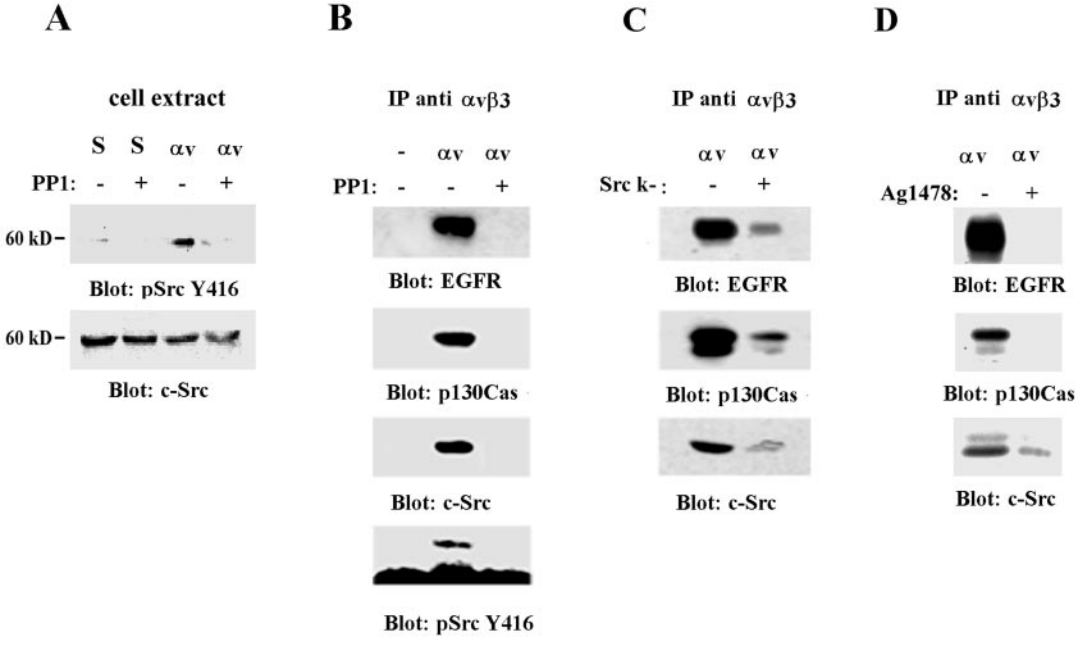



FIG. 3. **Assembly of integrin-EGF receptor macromolecular complex is dependent on c-Src and EGF receptor kinase activity.** *A*, ECV304 cells were detached from culture dishes and plated for 30 min on dishes coated with mAb L230 to the $\alpha_v$ integrin subunit in the presence or absence of 5 $\mu$M c-Src kinase inhibitor PP1. Cells were detergent extracted, and equal amounts of cell extracts were run on 10% SDS-PAGE and immunoblotted with antibodies to phosphorylated tyrosine 416 of c-Src (*pSrcY416*) (*upper panel*) or the c-Src protein (*lower panel*). *B*, ECV304 cells were plated for 5 min on dishes coated with PL and postcoated with mAb L230 to $\alpha_v$ integrin subunit in the presence or absence of 5 $\mu$M Src inhibitor PP1. Cell surface $\alpha_v\beta_3$ was bound as described in Figs. 1 and 2 and immunoprecipitated by the addition of protein A-Sepharose (*IP anti* $\alpha_v\beta_3$). Cell extracts were run as a control. Immunoblotting was performed with antibodies to EGF receptor (*EGFR*), p130Cas, c-Src, and phosphorylated tyrosine 416 of c-Src. *C*, ECV304 cells were transiently transfected for 40 h with control plasmid (−) or with pSGT Src K− (+), detached, plated on $\alpha_v$ antibodies for 5 min on dishes coated with PL, and postcoated with mAb L230 to $\alpha_v$ integrin ($\alpha_v$), and processed as in *A*. Immunoprecipitates were blotted with antibodies to EGF receptor (*top panel*), p130Cas (*middle panel*), and c-Src (*bottom panel*). *D*, ECV304 cells were plated for 5 min on dishes coated with PL and postcoated with mAb L230 to the $\alpha_v$ integrin subunit in the presence or absence of 250 nM tyrphostin AG1478 and processed as in *A*. Cells extracts were immunoprecipitated by the addition of protein A-Sepharose (*IP anti* $\alpha_v\beta_3$) or run as a control. Immunoblotting was performed with antibodies to EGF receptor, p130Cas, and c-Src. The data reported here are representative of three distinct experiments.

c-Src−/− fibroblasts (not shown). Therefore these data indicate that c-Src is needed for the assembly of the integrin-EGF receptor complex.

We also evaluated the role of EGF receptor kinase in modulating EGF receptor association with integrins by using tyrphostin AG1478, a specific inhibitor of EGF receptor kinase, in

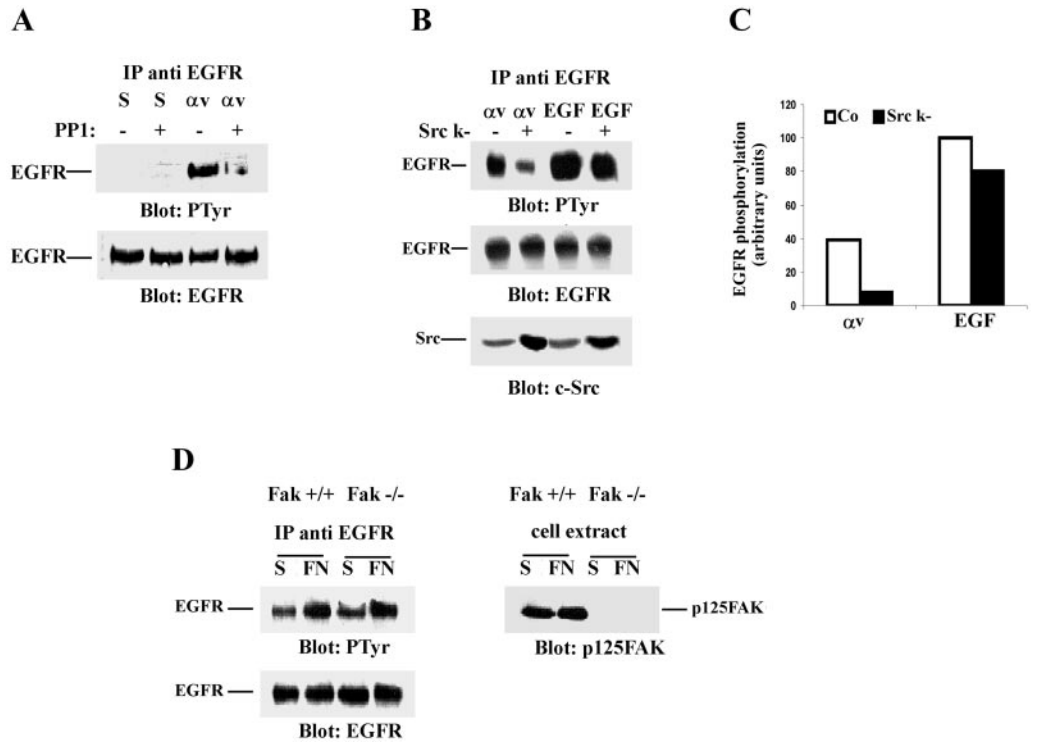



FIG. 4. **c-Src kinase activity is required to trigger EGF receptor phosphorylation in response to adhesion.** *A*, ECV304 cells were detached from culture dishes and plated for 30 min on dishes coated with mAb L230 to the $\alpha_v$ integrin subunit in the presence or absence of 5 $\mu$M c-Src kinase inhibitor PP1. EGF receptor was immunoprecipitated with mAb 8509 from ECV304 cells adherent to $\alpha_v$ antibodies, run on 6% SDS-PAGE, and blotted with antibodies to phosphotyrosine (*upper panel*) or to EGF receptor (*lower panel*). *B*, ECV304 cells were transiently transfected with control plasmid (−) or with pSGT Src K− (+), treated with 50 ng/ml EGF or detached and plated on $\alpha_v$ antibodies for 30 min (*αv*). Immunoprecipitated EGF receptor was blotted with antibodies to phosphotyrosine (*top panel*) or to EGF receptor (*middle panel*). Cell extracts were blotted with antibodies to the c-Src kinase protein (*bottom panel*). *C*, densitometric analysis of the experiment reported in *B*: control plasmid (*Co*), pSGTSrc K− (*Src k*−). Levels of EGF receptor phosphorylation are reported in arbitrary units. *D*, MEFs derived from Fak+/+ and Fak−/− embryos were detached from culture dishes and plated for 30 min on fibronectin-coated dishes. Immunoprecipitated EGF receptor (*EGFR*) was immunoblotted with antibodies to phosphotyrosine (*upper left panel*) or to EGF receptor (*lower left panel*); cell extracts were visualized for Fak expression with antibodies to p125Fak (*right panel*). The data reported here are representative of four distinct experiments.

the coimmunoprecipitation experiments. As shown in Fig. 3*D*, in the presence of tyrphostin AG1478, EGF receptors and p130Cas are not detectable in the integrin immunoprecipitate, whereas c-Src is still present, even if reduced, indicating that EGF receptor kinase activity is necessary for its ability to associate with integrins but is not required for association between integrins and c-Src.

*c-Src but Not p125Fak Kinase Is Required for Integrin-mediated Tyrosine Phosphorylation of EGF Receptor*—The data shown above indicate that c-Src kinase is required to trigger integrin/EGF receptor association. We then tested whether c-Src kinase is also necessary for integrin-dependent EGF receptor phosphorylation. When cells are exposed to the Src kinase inhibitor PP1, tyrosine phosphorylation of EGF receptors induced by integrin-mediated adhesion is strongly reduced (Fig. 4*A*). Similarly, expression of a kinase negative form of c-Src strongly affects integrin-dependent tyrosine phosphorylation of EGF receptor but only slightly modifies tyrosine phosphorylation in response to EGF (Fig. 4, *B* and *C*). Similar results were obtained using 10 or 50 ng/ml EGF (data not shown). This result indicates that c-Src kinase has a primary role in integrin-dependent EGF receptor tyrosine phosphorylation.

It is well established that p125Fak is regulated by integrin-mediated adhesion and is a good substrate for Src kinase (for review, see Refs. 3 and 8). The involvement of p125Fak kinase in integrin-dependent EGF receptor activation has been tested by comparing p125Fak−/− MEFs with wild type cells (38). p125Fak−/− cells plated on fibronectin show the same extent of EGF receptor phosphorylation as the wild type MEFs (Fig. 4*D*), indicating that p125Fak is not involved in EGF receptor phosphorylation. In addition, expression of a kinase-defective (CD2FakK454R) and of a tyrosine autophosphorylation mutant (CD2FakY397F) of p125Fak in ECV304 cells does not affect EGF receptor tyrosine phosphorylation after adhesion (data not shown), further supporting the conclusion that p125Fak is not required for integrin-mediated signaling leading to EGF receptor phosphorylation.

*p130Cas and the Integrin Cytoplasmic Domain Are Required for Integrin-dependent Phosphorylation of EGF Receptors*—The experiments reported above underline that EGF receptors transiently associate with integrins in combination with other molecules known to be involved in integrin-dependent signal transduction, such as the adaptor protein p130Cas (44). To dissect the molecular mechanisms of integrin-dependent EGF receptor phosphorylation, we tested whether the $\beta_1$ integrin cytoplasmic domain and p130Cas are relevant to this process. The contribution of the $\beta_1$ cytoplasmic domain was analyzed by using GD25 cells derived from $\beta_1$ integrin-null mice (45). These cells have been stably transfected with $\beta_1$ integrin ($\beta_1$A) or with $\beta_1$TR integrin mutant, lacking all of the cytoplasmic domain (37). Cells were plated on dishes coated with anti-$\beta_1$ integrin mAb TS2/16 in order to trigger only $\beta_1$ integrin-dependent signals. Upon adhesion to $\beta_1$ ligand, GD25$\beta$1A cells show induction of EGF receptor phosphorylation, but GD25$\beta$1TR cells do not (Fig. 5*A*). Therefore these data indicate that the $\beta_1$ cytoplasmic domain is required to trigger EGF receptor phosphorylation. Interestingly, in the same experimental conditions, EGF receptors are phosphorylated by EGF in both GD25$\beta$1A and GD25$\beta$1TR cells, indicating that EGF-induced

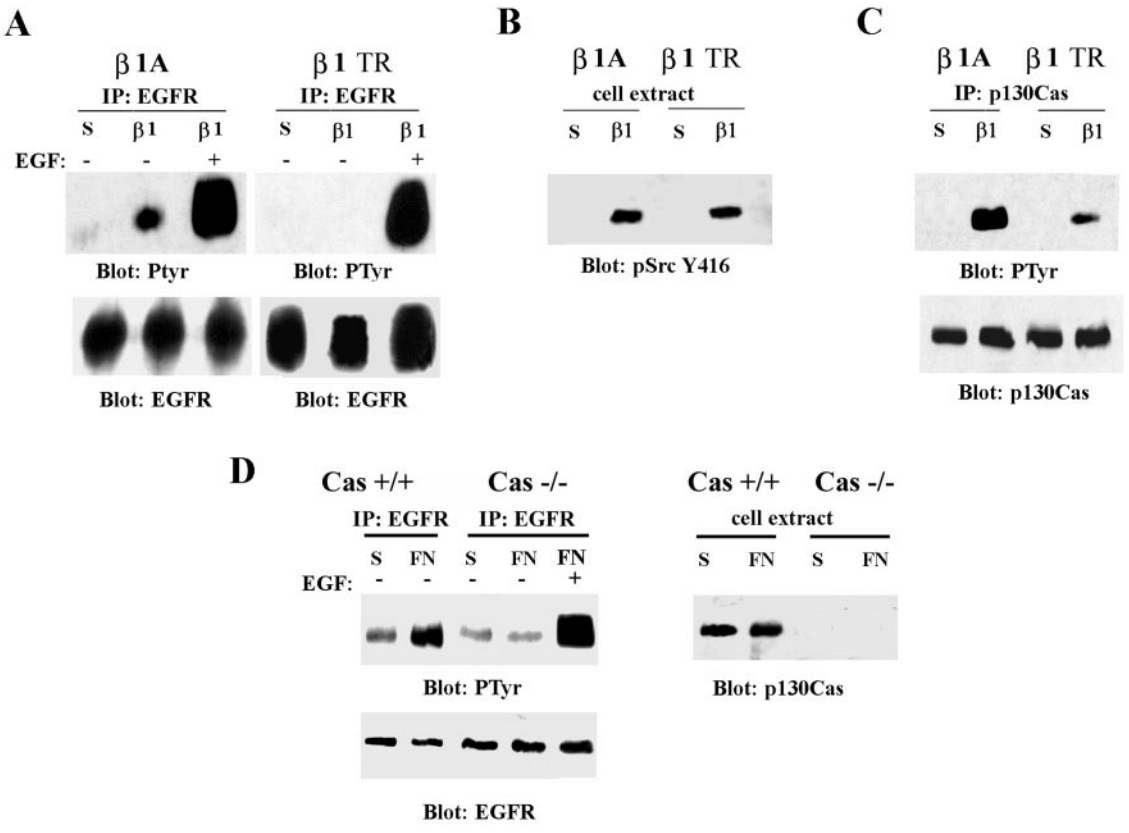



FIG. 5. **EGF receptor phosphorylation is dependent on $\beta_1$ integrin cytoplasmic domain and p130Cas.** *A*, GD25$\beta$1A and GD25$\beta$1TR cells were plated for 30 min on dishes coated with mAb TS2/16 to the $\beta_1$ integrin subunit in the presence or absence of 50 ng/ml EGF or kept in suspension (*S*). Cell extracts were immunoprecipitated by antibodies to EGF receptor (*EGFR*) or p130Cas, and the immunoprecipitates (*IP*) were blotted with antibody PY99 to phosphotyrosine (*upper panels*) and reblotted with polyclonal antibodies to the EGF receptor (*lower panels*). *B*, cell extracts of GD25$\beta$1A and GD25$\beta$1TR cells treated as in *A* were blotted with antibodies that specifically recognize c-Src when it is phosphorylated on its autophosphorylation site (*pSrcY416*). *C*, cell extracts from GD25$\beta$1A and GD25$\beta$1TR cells plated for 30 min on dishes coated with mAb TS2/16 to $\beta_1$ integrin were immunoprecipitated with antibodies to p130Cas. Immunoprecipitates were blotted with antibody PY99 to phosphotyrosine (*upper panel*) and reblotted with mAb to p130Cas (*lower panel*). *D*, cells derived from p130Cas+/+ and p130Cas−/− embryos were detached from culture dishes and plated for 30 min on fibronectin-coated dishes in the presence or in the absence of 50 ng/ml EGF or kept in suspension (*S*). Immunoprecipitated EGF receptor was immunoblotted with antibody PY99 to phosphotyrosine (*upper left panel*) or to EGF receptor (*lower left panel*) or to p130Cas (*right panel*). The data reported here are representative of three distinct experiments.

phosphorylation is independent of the presence of the $\beta_1$ integrin cytoplasmic domain and distinct from phosphorylation obtained by integrin-mediated adhesion.

In addition, using an antibody that recognizes phosphorylation of the tyrosine residue 416 in the Src kinase domain, we show that c-Src is phosphorylated on this tyrosine in both GD25$\beta$1A and GD25$\beta$1TR cells plated on $\beta_1$ integrin ligand, indicating that the lack of EGF receptor activation in GD25$\beta$1TR does not depend on defective c-Src activation (Fig. 5*B*). p130Cas has been described as a major c-Src-dependent phosphorylated protein upon cell/matrix interaction (44, 46–48). The extent of p130Cas tyrosine phosphorylation observed in GD25$\beta$1TR cells plated on $\beta_1$ ligand is strongly reduced compared with that obtained in GD25$\beta$1A cells, suggesting that the $\beta_1$ integrin cytoplasmic domain is involved in adhesion-dependent p130Cas tyrosine phosphorylation (Fig. 5*C*) and that, in the absence of the $\beta_1$ cytoplasmic domain, c-Src activation is not sufficient to trigger massive adhesion-dependent p130Cas phosphorylation.

Because p130Cas is a component of the integrin-EGF receptor complex, we investigated the role of this protein in EGF receptor phosphorylation using p130Cas-deficient cells. Wild type and p130Cas−/− MEFs were plated on fibronectin or kept in suspension, and EGF receptors were immunoprecipitated within 30 min of adhesion. p130Cas−/− fibroblasts were unable to trigger EGF receptor phosphorylation upon fibronectin adhesion (Fig. 5*D*), showing that the presence of the p130Cas molecule is required to trigger integrin-dependent EGF receptor phosphorylation.

*Integrin-mediated Adhesion Leads to Phosphorylation of Specific Residues on EGF Receptors*—We have shown previously that integrin-dependent EGF receptor phosphorylation is quantitatively lower than that obtained in response to EGF (33). To define which tyrosine residues are phosphorylated by cell-matrix adhesion we used MALDI-TOF mass spectrometry and antibodies to specific EGF receptor tyrosine residues. To analyze tyrosine residues phosphorylated in integrin-mediated adhesion, MALDI-TOF mass spectrometry analysis was performed on EGF receptors purified by affinity chromatography from cells plated on $\alpha_v$ ligand or kept in suspension. As shown in Table I tryptic peptides containing tyrosine residues 845 and 1068 are found phosphorylated in cells adherent to integrin ligand and not phosphorylated in cells kept in suspension, indicating that these two residues are targets of integrin-mediated adhesion. Interestingly, peptides containing tyrosine 1148 are not phosphorylated in response to integrin-mediated adhesion, but they are phosphorylated in response to EGF (data not shown), because tyrosine 1148 is a major EGF-dependent autophosphorylation site (49, 50).

These data were confirmed by using antibodies to specific tyrosine residues of EGF receptor. As shown in Fig. 6, tyrosine 1068 is phosphorylated strongly by cell-matrix adhesion (*top panel*), whereas tyrosine 1148 is not (*third panel* from *top*). As expected, both tyrosines are highly phosphorylated by EGF

TABLE I
*EGF receptor phosphotyrosines identified by MALDI-TOF mass spectrometry analysis after in-gel digestion*

| Tryptic peptides | Expected mass $MH^+$ | Measured mass $MH^+$ | |
|---|---|---|---|
| | | S | αv |
| $L^{837}$-$K^{851}$ | 1,630.807 | 1,630.691 | 1,630.862 |
| $L^{837}$-$K^{851}$ (P-$Y^{845}$) | 1,710.773 | | 1,710.836 |
| $Y^{1045}$-$K^{1075}$ | 3,398.617 | 3,398.329 | 3,398.572 |
| $Y^{1045}$-$K^{1075}$ (P-$Y^{1068}$) | 3,478.583 | | 3,478.242 |
| $G^{1137}$-$K^{1155}$ | 2,236.031 | 2,236.033 | 2,236.107 |
| $G^{1137}$-$K^{1155}$ (P-$Y^{1148}$) | 2,315.997 | | |

treatment. The use of two antibodies to phosphorylated tyrosine 1086 or 1173 shows also that these two residues are phosphorylated after adhesion (*second panel* from *top* and *second* from *bottom*), indicating that tyrosine 845 and 1068 are not the unique sites phosphorylated in response to integrin-mediated adhesion. Densitometric analysis of the Western blots shows that tyrosine 1068 is strongly phosphorylated after adhesion: the extent of phosphorylation was 70% of that found in cells treated with 10 ng/ml EGF. Kinetic analysis of phosphorylated tyrosines shows that phosphorylation of 1068 and 1086 peaks at 15 min and is slightly reduced within 30 min of adhesion. Tyrosine 1173 is also phosphorylated at 15 min, even if at a lesser extent, and its phosphorylation is down-regulated within 30 min. In addition, phosphorylation of all of these sites was abolished in presence of tyrphostin AG1478, suggesting that phosphorylation occurs via the EGF receptor kinase. It would also be possible that selective inhibition of tyrosine phosphatases contributes to increased EGF receptor tyrosine phosphorylation on specific sites in response to adhesion. To investigate this possibility, cells were plated for 15 min on matrix ligands to trigger EGF receptor phosphorylation, then the EGF receptor kinase was "frozen" by treatment with tyrphostin AG1478; phosphorylation was analyzed at 30 and 60 min of adhesion. AG1478 treatment abolishes phosphorylation of tyrosine 1068 and 1173, indicating that the EGF receptor kinase activity, rather than a decreased phosphatase activity, is required to maintain tyrosine phosphorylation of these two residues (data not shown). These data show that after integrin-dependent cell-matrix adhesion, specific EGF receptor tyrosine residues become phosphorylated and that they do not correspond to all of the major sites previously shown to be phosphorylated in response to EGF.

*Integrin-dependent Adhesion Increases the Amount of Cell Surface-exposed EGF Receptor*—The integrin-EGF receptor complex can be immunoprecipitated from the cell surface either with antibodies to integrin subunits or to EGF receptors (33). When the EGF receptor is immunoprecipitated, the amount of EGF receptor recovered from the surface of cells plated on $\alpha_v$ ligand is higher than that obtained from cells plated on PL (Fig. 7*A*). In the presence of c-Src kinase inhibitor PP1, however, the level of EGF receptor decreases to that observed in cells plated on PL (Fig. 7*A*). Densitometric analysis show a 50% increase of EGF receptor level in cells plated on integrin ligands (Fig. 7*B*). These data suggest that integrin-mediated adhesion increases the EGF receptor detectable on the cell surface. The results obtained by immunoprecipitation were confirmed by immunoelectron microscopy analysis. Gold particle counting increases in cells plated on $\alpha_v$ ligand compared with cells plated on PL and is reduced in the presence of c-Src inhibitor PP1 (Fig. 7*B*). Therefore these data show that cell-matrix adhesion induces a c-Src-dependent increase in the EGF receptor level on the cell surface.

## DISCUSSION

In this study we dissect the molecular mechanisms leading to integrin-dependent tyrosine phosphorylation of EGF receptors, which occurs upon cell-matrix adhesion. We show that: 1) after adhesion, integrins and EGF receptors transiently associate in a macromolecular complex, which contains the c-Src kinase and the adaptor molecules p130Cas and Crk; 2) c-Src and EGF receptor kinases are both required for association of integrins and EGF receptors; 3) complex formation is required for EGF receptor phosphorylation; 4) the $\beta_1$ integrin cytoplasmic domain and the adaptor molecule p130Cas are additional elements required to trigger integrin-dependent EGF receptor phosphorylation; 5) integrins induce a pattern of EGF receptor phosphorylation distinct from that induced by EGF.

Formation of integrins and growth factor receptor macromolecular complexes has been suggested by co-clustering and immunofluorescence experiments (21, 31, 51) as well as by direct coimmunoprecipitation (19, 22, 28, 33, 51, 52). While most of these complexes were detected in response to growth factor stimulation, we show here that in the absence of growth factors, integrins dynamically associate with the EGF receptor in response to cell/matrix interaction. The integrin-EGF receptor macromolecular complex is specifically localized at the cell membrane and is a dynamic structure that is detectable at 5 min of cell adhesion and rapidly down-regulated.

In addition to integrins and the EGF receptor, this complex also includes molecules involved in signal transduction, such as c-Src, p130Cas, and Crk. Our data show that c-Src is activated after adhesion and phosphorylated on tyrosine residue 416 in the activation loop, suggesting that c-Src is activated through an autophosphorylation mechanism. The activated form of c-Src is present in the integrin-EGF receptor complex, suggesting a role for this kinase in complex assembly. This was confirmed by using a pharmacological inhibitor of Src kinase activity and a kinase-defective construct, which both prevent complex assembly. Inhibition of c-Src kinase activity blocks association of integrins, EGF receptor, p130Cas, and c-Src, demonstrating that c-Src catalytic activity is required to build up the macromolecular complex. In contrast, when EGF receptor kinase activity is blocked by the specific tyrphostin AG1478, integrins are still able to associate with c-Src, even if at a reduced extent, but they lose their ability to coimmunoprecipitate EGF receptor and p130Cas. Therefore these data indicate that EGF receptor tyrosine kinase activity is necessary for its association with integrins but is not required for association between integrins and c-Src. Taken together these data suggest that after adhesion, a hierarchy of events takes place, leading first to integrin-dependent c-Src activation and then to c-Src kinase-dependent recruitment of p130Cas and EGF receptors in the macromolecular complex.

p125Fak kinase, which is known to associate with p130Cas and c-Src (10, 53–55), is not present in the integrin-EGF receptor complex. This finding is consistent with our result that p125Fak is not required for tyrosine phosphorylation of EGF receptors in response to integrins, as shown using cells derived from p125Fak knock-out mice or p125Fak dominant negative mutants. Recently Sieg *et al.* (56) reported the ability of p125Fak to associate in a complex with EGF receptors. The lack of p125Fak coprecipitation in our experiments is likely to be the result of the different experimental conditions used. These authors detected this association in stable adherent cells only in response to EGF, whereas our analysis was performed in the absence of EGF on cells in the early phases of integrin-mediated adhesion.

As discussed above c-Src catalytic activity is required for integrin-EGF receptor macromolecular complex formation. In addition c-Src catalytic activity is also critical for EGF receptor phosphorylation, indicating that the macromolecular complex is required to trigger EGF receptor phosphorylation. A central

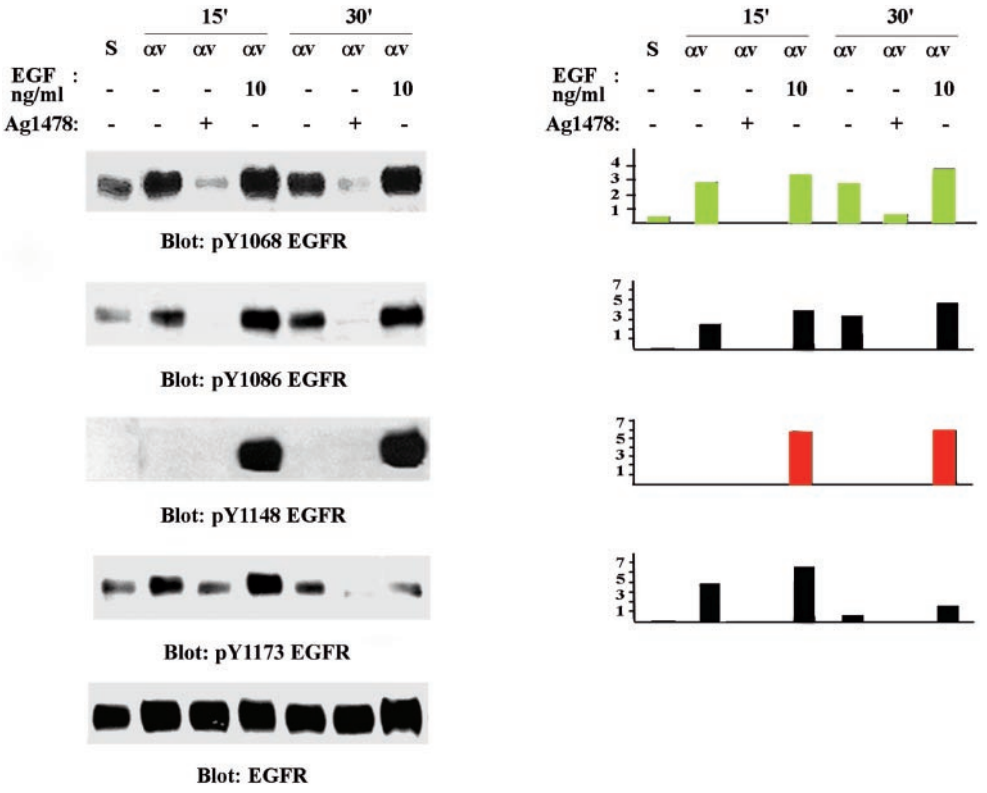


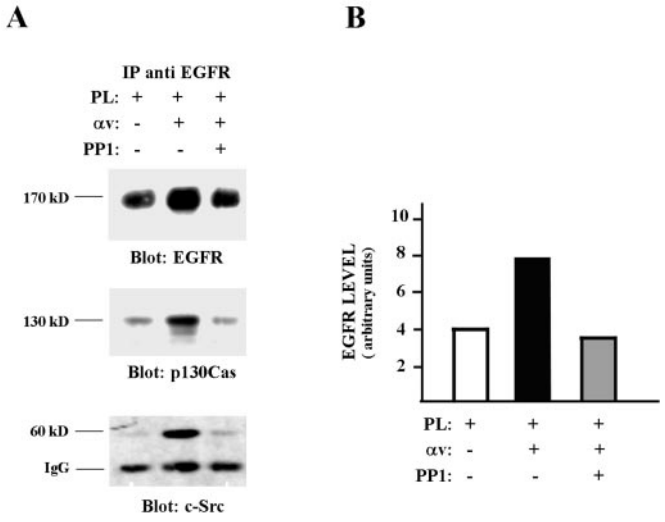

FIG. 6. **EGF receptor tyrosine 1068, 1086, and 1173 are phosphorylated by integrin-dependent adhesion.** ECV304 cells were kept in suspension (*S*) or plated for 15 and 30 min on dishes coated with mAb L230 to the $\alpha_v$ integrin subunit in the presence or absence of 10 ng/ml EGF or 250 nM EGF receptor kinase inhibitor tyrphostin AG1478. Cell extracts were subjected to 6% SDS-PAGE and blotted with antibodies that specifically recognize phosphorylated tyrosine 1068 (*pY1068 EGFR*), 1086 (*pY1086 EGFR*), 1173 (*pY1173 EGFR*) or 1148 (*pY1148 EGFR*). The same blots were reblotted with antibodies to EGF receptor for normalization (*bottom left panel*). Densitometric analysis of each experiment is shown on the *right*. The data reported here are representative of three distinct experiments.

role for c-Src in integrin signaling has been underlined by several experiments (for review, see Ref. 57). Fibroblasts derived from Src-deficient mice show delayed spreading on fibronectin (58) or vitronectin (59), suggesting that c-Src modulates integrin-dependent adhesion and spreading by regulating the strength or dynamics of integrin/cytoskeleton interactions. In addition, c-Src is involved in focal adhesion formation and disassembly (60), and the triple mutant SYF(Src−/−, Yes−/−, and Fyn−/−) cells are deficient in fibronectin-induced tyrosine phosphorylation of focal adhesion protein (61). Kinase activity of c-Src has also been shown to associate with $\alpha_v\beta_3$ integrin in osteoclasts and melanoma cells (62), indicating that integrins and c-Src function in association. Consistent with our data, c-Src has also been shown to be involved in integrin-dependent RON phosphorylation (63).

In addition to c-Src kinase, the $\beta_1$ integrin cytoplasmic domain as well as p130Cas protein are additional elements required for integrin-dependent EGF receptor phosphorylation. The $\beta_1$ integrin mutant lacking the cytoplasmic domain does not trigger EGF receptor phosphorylation, indicating that the cytoplasmic domain is required to induce this event. Interestingly, integrin heterodimers in which the integrin $\beta_1$ subunit cytoplasmic domain is truncated still activate c-Src, thus suggesting that either the $\alpha$ subunit or the extracellular part of the molecule is required for this function. Previous reports have indeed indicated a role for specific $\alpha$ subunits in Src family kinase activation (5, 12). $\beta_1$ integrins lacking the cytoplasmic domain, however, show an impaired ability to phosphorylate p130Cas. p130Cas adaptor protein is required for integrin-dependent EGF receptor phosphorylation, as shown by using fibroblasts derived from p130Cas−/− mice. p130Cas phosphorylation and localization to focal adhesions has been shown to be dependent on c-Src (47, 48). Our data show also that the cytoplasmic domain of $\beta_1$ integrin is required for integrin-dependent p130Cas phosphorylation, suggesting that the $\beta_1$ cytoplasmic domain is crucial for correct membrane targeting of p130Cas and its assembly in the integrin-EGF receptor complex.

As shown above, the integrin-EGF receptor macromolecular complex is a dynamic structure that is rapidly down-regulated from the cell surface. Integrin-dependent EGF receptor phosphorylation takes place at the same time as complex assembly, but it is more persistent, remaining high within 30 min of adhesion, even when the complex is disassembled. The basis of this phenomenon is unclear at present. Interestingly, an EGF receptor new activation mechanism has recently been shown, which consists in ligand-independent rapid and extensive propagation of receptor phosphorylation over the entire cell after focal stimulation (64).

MALDI-TOF mass spectrometry analysis and the use of phospho-specific antibodies led us to detect integrin-dependent phosphorylation of four EGF receptor tyrosines, namely the 845, 1068, 1086 and 1173 residues. Interestingly, tyrosine 1148 is not phosphorylated in response to adhesion. This tyrosine residue, however, is a major site that is phosphorylated in response to EGF (49, 50), and we detected its phosphorylation by both mass spectrometry and phospho-specific antibody staining (Fig. 6). These data thus strongly indicate that integrins induce a pattern of EGF receptor phosphorylation distinct from that induced by EGF. The fact that tyrosine 1148 is not phosphorylated in response to adhesion reflects a distinct mechanism of phosphorylation of EGF receptors in response to adhesion rather than to EGF. This hypothesis is also supported by the finding that c-Src activity is required for integrin-de-




C

| | G/PM |
|---|---|
| PL | 147 |
| PL+αv | 216 |
| PL+αv+PP1 | 127 |

FIG. 7. **Adhesion to $\alpha_v$ antibodies increases EGF receptor level on the cell surface.** *A*, ECV304 cells were plated for 5 min on dishes coated with PL and postcoated with mAb L230 to the $\alpha_v$ integrin subunit in the presence or absence of 5 $\mu$M Src inhibitor PP1. mAb 8509 to EGF receptor was then added on the cells, which were incubated further for 30 min at 4 °C before detergent extraction. Cells extracts were immunoprecipitated by the addition of protein A-Sepharose (*IP anti EGFR*), and the immunoprecipitates were blotted with antibodies to EGF receptor (*EGFR*), p130Cas, and c-Src. *B*, densitometric analysis of the experiment reported in *A*; EGF receptor levels are reported in arbitrary units. *C*, ECV304 cells treated in the same conditions as in *A* were fixed and frozen in liquid nitrogen. Ultrathin cryosections were immunostained with mouse monoclonal anti hEGFR followed by a rabbit anti-mouse bridging antibody (DAKO) and 15-nm protein A-gold. Sections were examined with a Zeiss EM 902 electron microscope. The data reported here are representative of three distinct experiments.

pendent EGF receptor phosphorylation but not for ligand-dependent phosphorylation. The mechanisms responsible for the lack of phosphorylation of tyrosine 1148 are unclear and could be the result of either masking of this specific site within the complex or the presence of an active site-specific phosphatase.

Tyrphostin AG1478 abolishes phosphorylation of 1068, 1086, and 1173, indicating that EGF receptor kinase plays a primary role in this phosphorylation. Phosphorylation of tyrosine residues depends on a balance between kinase and phosphatase activity. When EGF receptor kinase was frozen with tyrphostin AG1478 after phosphorylation has occurred (the addition of tyrphostin at 15 min of adhesion), phosphorylation of tyrosine 1068 and 1173 was rapidly lost, indicating that increased EGF receptor kinase activity rather than decreased phosphatase activity controls the phosphorylation process. Nevertheless EGF receptors are known to interact with tyrosine phosphatase SHIP2 (65), which has also been recently reported as a p130Cas interactor (66). Therefore we cannot exclude that protein-tyrosine phosphatases that are present in the complex could be somehow regulated in the integrin-dependent EGF activation process.

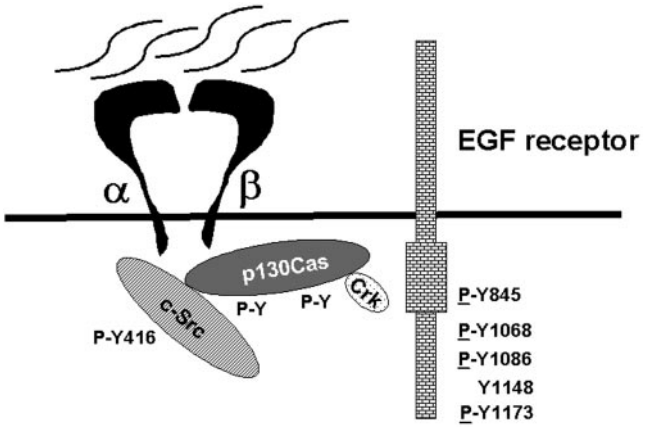



FIG. 8. **Model of integrin-dependent EGF receptor phosphorylation.** In response to cell-matrix adhesion, c-Src kinase is phosphorylated on tyrosine 416 (*PY416*) and associates in a complex with integrins, p130Cas, Crk, and EGF receptor. As a consequence of cell adhesion, EGF receptor is phosphorylated on tyrosine 845, 1068, 1086, and 1173, but not on tyrosine 1148.

c-Src kinase activity regulates EGF receptor phosphorylation either because it is required for the assembly of the integrin-EGF receptor complex, which, in turn, allows EGF receptor phosphorylation, or because it directly regulates the EGF receptor kinase. Analysis of the phosphorylated EGF receptor sites shows that tyrosine 845 is phosphorylated upon adhesion-mediated receptor activation. Previous data showed that tyrosine 845 can be phosphorylated by c-Src *in vitro* (67) and *in vivo* in cells overexpressing active c-Src kinase (68). Our data, showing that c-Src is required for integrin-dependent EGF receptor phosphorylation, suggest the possibility that tyrosine 845 can be directly phosphorylated by c-Src.

Therefore we propose a model in which, after cell-matrix adhesion, c-Src kinase is activated, associates in a complex with integrins, p130Cas, and EGF receptors, leading to phosphorylation of EGF receptors at specific tyrosine residues, such as 1068, 1086, and 1173, but not the 1148 site (Fig. 8).

Endocytosis of growth factor receptors is an important step in growth factor activity, known to regulate downstream signaling (69, 70). An interesting observation emerging from our data is that in cells adherent to integrin ligands, the amount of EGF receptor localized on the cell membrane is significantly increased, suggesting that in the early phases of integrin-dependent adhesion, EGF receptors are stabilized on the plasma membrane. This event occurs in the earliest phases of cell adhesion, indicating that the increased expression observed cannot be the result of increased transcription. Additional experiments will clarify whether this event might depend on reduced internalization or increased recycling and whether it can play any role in EGF receptor internalization process. Nevertheless this increase is abolished when c-Src kinase is inhibited, a condition shown to prevent complex formation and EGF receptor phosphorylation, thus strongly suggesting that integrin-EGF receptor complex formation triggers specific events responsible for increased EGF receptor exposure on the cell surface.

Integrins have been shown to potentiate signaling pathways in response to insulin, EGF, platelet-derived growth factor, fibroblast growth factor, and vascular endothelial growth factor (19–24, 28; for review, see Ref. 29). The ability of integrins to transactivate EGF receptors, as reported in our work, can thus represent a molecular mechanism at the basis of this phenomenon. Indeed integrin-dependent growth factor receptor activation is not restricted to the EGF receptor. It has been shown, in fact, that cell/matrix interaction stimulates phosphorylation of hepatocyte growth factor receptors (30, 32), platelet-derived

growth factor β receptors (31), and RON kinase (63), suggesting that activation of growth factor receptors in the absence of their specific ligands can be a broadly used mechanism in adhesion-mediated signaling.

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