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 receptor, 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 α and β 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 Ca²⁺ influx, cytoplasmic alkalization, potassium channel activation, tyrosine phosphorylation of cytoplasmic proteins, and activation of the mitogen-activated protein (MAP) kinases ERK-1 and ERK-2. 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 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 β1 and β3 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 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.

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Integrin-dependent activation of the small GTPase Rac (for...
review, see Refs. 14 and 15) has also been proposed recently as an additional mechanism to regulate adhesion-dependent events, such as integrin activation ofJun NH2-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, αvβ3 integrin has been shown to synergize with different growth factor receptors. αvβ3 integrin occupancy by its matrix ligand is required for full tyrosine phosphorylation of insulin and platelet-derived growth factor β receptors and their binding to several signaling molecules such as insulin receptor substrate 1, phospholipase Cγ, Ras GAP, the p85 subunit of phosphatidylinositol 3-kinase and the tyrosine phosphatase SH2P2 (19, 22). In endothelial cells, moreover, αvβ3 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 β3 integrin subunit (purchased from ATCC), mAb L230 to the αv integrin subunit (from ATCC), mAb B212 to the β3 subunit, and the polyclonal antibody to the β3 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 β2/5Fak 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-aminoo-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 HB-8508 (purchased from ATCC), mAb L230 to the activated form of EGF receptor were prepared from BIOSOURCE International. Protein A-Sepharose beads were then added to 3 μg 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, and 10% acetic acid in 50% 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 μl of the peptide mixtures were applied to a target disc and allowed to air dry. Subsequently, 0.5 μl of matrix solution (1% w/v a-cyano-4-hydroxycinnamic acid in 50% ammonium bicarbonate, 0.1% trifluoroacetic acid) was applied to the matrix and again allowed to air 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 PepIdent applet program were used.

Immunoelectron Microscopy—For immunoelectron microscopy, 10% gelatin-embedded, 2.3 μm sucrose-infused blocks of aldehyde-fixed ECV304 cells were cut in liquid nitrogen. Ultrathin cryosections were obtained with a Reichert-Jung Ultracut E with FC4 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.
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 αv integrin subunit. mAb B212 to the β3 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 αvβ3) 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 β1 integrin. Materials coimmunoprecipitated with β1 integrin were run on gel and blotted, respectively, with the antibodies to EGF receptor, p130Cas, and the β1 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 β1 integrin (33). To investigate the molecular nature of the complex between integrins and the EGF receptor, we immunoprecipitated αvβ3 integrins from ECV304 cells adherent to αv ligand and performed Western blotting experiments. Fig. 1A (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 integrin-EGF receptor complex identified in ECV304 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. 1B).

Integrin-EGF Receptor Macromolecular Complex Is Transiently Assembled.—To investigate the kinetics of macromolecular complex formation, ECV304 cells were plated on dishes coated with αv 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. 2A) 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. 2B), 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. 3A). In ECV304 cells plated on αv 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. 3B, 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. 3A). After PP1 treatment, EGF receptors, p130Cas, and c-Src were not detectable in the immunoprecipitates of αvβ3 integrin (Fig. 3B), 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. 3C). Similar results were obtained in
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 c-Src−/− fibroblasts (not shown). Therefore these data indicate that c-Src is needed for the assembly of the integrin-EGF receptor complex.
the coimmunoprecipitation experiments. As shown in Fig. 3D, in the presence of tyrophostin 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. 4A). 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. 4B, C, and D). 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 β1 integrin cytoplasmic domain and p130Cas are relevant to this process. The contribution of the β1 cytoplasmic domain was analyzed by using GD25 cells derived from β1 integrin-null mice (45). These cells have been stably transfected with β1 integrin (β1A) or with β1,TR integrin mutant, lacking all of the cytoplasmic domain (37). Cells were plated on dishes coated with anti-β1 integrin Ab TS2/16 in order to trigger only β1 integrin-dependent signals. Upon adhesion to β1 ligand, GD25/β1A cells show induction of EGF receptor phosphorylation, but GD25/β1TR cells do not (Fig. 5A). Therefore these data indicate that the β1 cytoplasmic domain is required to trigger EGF receptor phosphorylation. Interestingly, in the same experimental conditions, EGF receptors are phosphorylated by EGF in both GD25/β1A and GD25/β1TR cells, indicating that EGF-induced
phosphorylation is independent of the presence of the β1 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 fibroblasts and p130Cas phosphorylation. We have previously shown 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, MALDI-TOF mass spectrometry analysis was performed on EGF receptors purified by affinity chromatography 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 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.

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. 5D), 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, 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 αIICG 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 1148 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.
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 tyrophostin 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 tyrophostin 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 αv ligand is higher than that obtained from cells plated on PL (Fig. 7A). 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. 7A). Densitometric analysis show a 50% increase of EGF receptor level in cells plated on integrin ligands (Fig. 7B). 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 αv ligand compared with cells plated on PL and is reduced in the presence of c-Src inhibitor PP1 (Fig. 7B). 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 β3 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 tyrophostin 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

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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/H11002/H11002, Yes/H11002/H11002, and Fyn/H11002/H11002) 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 αvβ3 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 αv integrin cytoplasmic domain as well as p130Cas protein are additional elements required for integrin-dependent EGF receptor phosphorylation. The β1 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 αvβ1 subunit cytoplasmic domain is truncated still activate c-Src, thus suggesting that either the α subunit or the extracellular part of the molecule is required for this function. Previous reports have indeed indicated a role for specific α subunits in Src family kinase activation (5, 12). β1 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 β1 integrin is required for integrin-dependent p130Cas phosphorylation, suggesting that the β1 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-
Mechanisms of Integrin-dependent EGF Receptor Phosphorylation

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D

\[ \text{IP anti EGF/} \]
\[ \begin{array}{c}
\text{PL: + + + +} \\
\text{αv: - + + +} \\
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\[ \begin{array}{c}
\text{170 kD} \\
\text{130 kD} \\
\text{60 kD} \\
\text{IgG}
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\[ \text{Blot: EGF/} \]
\[ \text{Blot: p130Cas} \]

\[ \text{EGFR LEVEL (arbitrary units)} \]

\[ \begin{array}{c}
\text{PL: + + + +} \\
\text{αv: - + + +} \\
\text{PP1: - - + +}
\end{array} \]

\[ \begin{array}{c}
\text{4} \\
\text{2} \\
\text{1} \\
\text{0}
\end{array} \]

FIG. 7. Adhesion to α, 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 α, integrin subunit in the presence or absence of 5 µM 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 EGF/), 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.

Fig. 8. Model of integrin-dependent EGF receptor phosphorylation. In response to cell-matrix adhesion, c-Src kinase is phosphorylated on tyrosine 416 (PY116) and associates in a complex with integrins, p130Cas, Crk, and EGF receptor. As a consequence of cell adhesion, EGF receptor is phosphorylated on tyrosines 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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