Integrin Function: Molecular Hierarchies of Cytoskeletal and Signaling Molecules

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Abstract. Integrin receptors play important roles in organizing the actin-containing cytoskeleton and in signal transduction from the extracellular matrix. The initial steps in integrin function can be analyzed experimentally using beads coated with ligands or anti-integrin antibodies to trigger rapid focal transmembrane responses. A hierarchy of transmembrane actions was identified in this study. Simple integrin aggregation triggered localized transmembrane accumulation of 20 signal transduction molecules, including RhoA, Rac1, Ras, Raf, MEK, ERK, and JNK. In contrast, out of eight cytoskeletal molecules tested, only tensin coaccumulated. Integrin aggregation alone was also sufficient to induce rapid activation of the JNK pathway, with kinetics of activation different from those of ERK. The tyrosine kinase inhibitors herbimycin A or genistein blocked both the accumulation of 19 out of 20 signal transduction molecules and JNK- and ERK-mediated signaling. Cytochalasin D had identical effects, whereas three other tyrosine kinase inhibitors did not. The sole exception among signaling molecules was the kinase pp125FAK which continued to coaggregate with αβ3 integrins even in the presence of these inhibitors. Tyrosine kinase inhibition also failed to block the ability of ligand occupancy plus integrin aggregation to trigger transmembrane accumulation of the three cytoskeletal molecules talin, α-actinin, and vinculin; these molecules accumulated even in the presence of cytochalasin D. However, it was necessary to fulfill all four conditions, i.e., integrin aggregation, integrin occupancy, tyrosine kinase activity, and actin cytoskeletal integrity, to achieve integrin-mediated focal accumulation of other cytoskeletal molecules including F-actin and paxillin. Integrins therefore mediate a transmembrane hierarchy of molecular responses.

INTEGRIN receptors for extracellular matrix molecules play central and complex roles in cell interactions. They mediate cell adhesion, migration, and invasion, but they also have a multitude of intracellular effects on the organization of the actin-containing cytoskeleton as well as roles in a variety of signaling processes (for reviews see Hynes, 1992; Sastry and Horwitz, 1993; Juliano and Haskill, 1993; Gumbiner, 1993; Pavalko and Otey, 1994; Schaller and Parsons, 1994; Shattil et al., 1994a; Clark and Brugge, 1995). A complex series of steps leads from initial integrin interactions with an extracellular ligand to transmembrane effects on the localization of cytoskeletal molecules or signaling molecules, to the activation of signaling pathways, and to eventual regulation of gene expression. Identifying distinct mechanisms of integrin responses to extracellular stimuli and patterns in the classes of responding molecules will be crucial for understanding how integrins function.

Extracellular interactions of integrins leading to specific cytoplasmic responses have been studied in many cell types, particularly fibroblasts and platelets. Besides providing insights into the activation of integrin ligand-binding functions, studies of platelets have defined steps in the intracellular response to ligation of the major platelet integrin αIIbβ3. Occupancy by monomeric soluble ligand has no apparent effects, whereas divalent ligand can stimulate Syk activation (Clark et al., 1994); however, two subsequent waves of phosphorylation present in normal platelet aggregation require immobilized ligand, platelet activation, and/or platelet–platelet aggregation (Shattil et al., 1994a). For example, phosphorylation of 50–72 kD and 140-kD proteins of unknown function occur on immobilized fibrinogen, while other responses such as FAK (focal adhesion kinase) phosphorylation require platelet–platelet aggregation (Huang et al., 1993; Shattil et al., 1994b). Platelet aggregation with immobilized ligand leads to major increases in association of αIIbβ3 with large cytoskeletal complexes. This anchorage of integrin to the platelet cytoskeleton occurs in parallel with immobilization of a variety of kinases such as c-Src (Shattil et al., 1994a).
terpretation of these findings is that ligand occupancy and limited aggregation of the \(\alpha_{m}\beta_{3}\) integrin provides a very rapid trigger for the first step of the intracellular response, and that the next steps are induced by the subsequent aggregation of these integrins and cells due to immobilized ligand or platelet-to-platelet aggregation. However, this scenario is difficult to test directly in platelets due to their small size.

Fibroblast systems have provided complementary information. A variety of signaling responses including calcium influx and changes in cytoplasmic pH have been identified (Schwartz et al., 1991; Leavesley et al., 1993). The phosphorylation on tyrosine residues of focal adhesion kinase (also termed pp125\(^{\text{FAK}}\) or FAK; for review see Schaller and Parsons, 1994) has been intensively studied. FAK phosphorylation can be induced by antibody-induced clustering of intact integrins or even of molecular chimeras containing only the intracellular domain of the \(\beta_{3}\) integrin subunit (Kornberg et al., 1991; Akiyama et al., 1994). Integrin ligation can also trigger activation of certain MAP kinases, specifically extracellular signal-regulated kinase (ERK)\(^{1}\) members (Chen et al., 1994; Morino et al., 1994; Schlaepfer et al., 1994). Other studies have linked ERK activation to growth factor response pathways, e.g., EGF receptor activation of growth. Other types of MAP kinases besides ERK1 and ERK2 have distinct pathways and functions (Davis, 1994; Oehlen and Cross, 1994). For example, SAPK (stress-activated protein kinase)/JNK (JUN kinase) is not activated by FGF receptors in most cell types, but instead becomes activated after treatment of cells by inflammatory cytokines or after stresses such as UV irradiation (Derijard et al., 1994). Comparisons of possible roles of integrins in activating these distinct signaling pathways should provide insights into the relationship of integrin ligation and signal transduction to the nucleus.

Cytoskeletal responses to integrin ligation can be analyzed using beads coated with a variety of molecules that interact with integrins. Fibronection-coated beads induce the local accumulation of a variety of cytoskeletal molecules, including vinculin, talin, \(\alpha\)-actinin, and F-actin (Grinnell and Geiger, 1986; Mueller et al., 1989; Plopper and Ingber, 1993; Lewis and Schwartz, 1995). The initial integrin response in fibroblasts can be dissected into three parts using soluble ligands vs immobilized multivalent ligands, and antibodies that can induce integrin aggregation either with or without effects on adhesive function (Miyamoto et al., 1995). There are distinct effects on integrin cytoskeletal function mediated by ligand occupancy alone, by receptor aggregation alone, or by a combination of occupancy and aggregation. Ligand occupancy with soluble, monovalent ligand triggers fibroblast integrin localization to preexisting focal contacts, but does not trigger FAK phosphorylation. Direct integrin aggregation without ligand occupancy mediated by noninhibitory monovalent antibodies triggers FAK phosphorylation as well as the local accumulation of FAK and the cytoskeletal protein tensin in a focal location immediately adjacent to anti-integrin coated beads. However, a combination of integrin aggregation and occupancy by soluble ligand or antifunctional antibodies triggers a synergistic response involving the accumulation of six other cytoskeletal proteins including talin and F-actin (Miyamoto et al., 1995).

In this study, we explore the hypothesis that the extracellular triggering of an integrin response involves a series of specific stages or hierarchies of protein interactions. A substantial number of potential intermolecular interactions are already known, including evidence for the binding of talin, \(\alpha\)-actinin, and FAK to the \(\beta_{3}\) integrin cytoplasmic domain (Horwitz et al., 1986; Otey et al., 1990; Schaller and Parsons, 1994; Lewis and Schwartz, 1995). In addition, there is accumulating evidence to suggest the existence of protein–protein interactions among cytoskeletal proteins and a variety of signaling molecules (for a review see Clark and Brugge, 1995). Because so many proteins can potentially interact with each other based on in vitro studies, it is important to determine whether there are specific patterns or hierarchies of interactions induced by integrins within the cytoplasmic milieu.

In the present study, we have compared a series of over 20 proteins implicated in various signal transduction events with a variety of cytoskeletal proteins using a bead-binding system that permits local experimental triggering of transmembrane responses. These responses were then dissected using inhibitors of tyrosine phosphorylation and actin integrity to search for differential requirements. The goal was to identify hierarchies of interaction, since so many molecules appear to contribute to integrin-induced complexes (e.g., see molecules already identified by Burridge et al., 1988; Plopper and Ingber, 1993; Miyamoto et al., 1995).

Using this approach, we have classified 32 signal-transduction and cytoskeletal proteins into at least five distinct groups depending on their ability to form juxtamembrane complexes in response to signals involving (a) the state of integrin receptor aggregation, (b) integrin occupancy, (c) cytoplasmic tyrosine phosphorylation, and (d) actin cytoskeletal integrity. Of these stimuli involved in complex formation, three appear to be required for integrin-mediated activation of the ERK and JNK pathways, which are thought to be common intermediaries in many membrane-nuclear signaling processes. Our studies provide the first detailed comparative analysis of the requirements for integrin-mediated regulation of families of signal transduction and cytoskeletal molecules.

Materials and Methods

Cell Culture and bead Assays

Human foreskin fibroblasts were cultured in DME medium supplemented with 10% heat-inactivated FBS (HyClone, Logan, UT), 1 mM glutamine, 50 \(\mu\)g/ml streptomycin, and 50 U/ml penicillin. The cells were a generous gift from Dr. Steve Alexander (Bethesda Research Laboratories, Gaithersburg, MD), and were used at cell passages 9–18.

Latex beads (mean diameter 11.9 \(\mu\)m, Sigma Chem. Co., St. Louis, MO) were coated with ligand or antibody and incubated with cells exactly as described previously for the experimental induction of focal integrin clustering and transmembrane accumulation of cytoskeletal molecules (Miyamoto et al., 1995). After 20 min incubation with beads, cells were fixed in PBS with 4% paraformaldehyde and 5% sucrose, and analyzed by immunofluorescence microscopy as described (LaFlamme et al., 1992; Miyamoto et al., 1995) using a Nikon HFX-II microscope equipped for fluorescein and rhodamine fluorescence. In experiments using mouse anti-\(\beta_{2}\) K20 antibody on beads, an excess of rat monoclonal anti-mouse IgG2a

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1. Abbreviations used in this paper: ERK, extracellular signal-regulated kinase; FAK, focal adhesion kinase; JNK, Jun kinase.
antibody (50 μg/ml) was included at each step to block cross-reactivity of the FITC-labeled secondary rat monoclonal anti-mouse IgG1 antibodies.

**Immunological Reagents**

Antibodies to integrins used to coat the latex beads were rat monoclonal antibodies mAb 11 and mAb 16 to the α5 integrin subunit and mAb 13 to the β1 integrin subunit (Akiyama et al., 1989; LaFlamme et al., 1992). Mouse monoclonal antibody K20 to the β1 integrin subunit was from AMAC (Westbrook, ME). The following polyclonal antibodies were purchased and shown to be monospecific by Western immunoblotting using goat anti-rabbit IgG conjugated with peroxidase (Pierce Co., Rockford, IL); c-Src, c-Fyn, c-Csk, c-Lyn, PLC-γ, PTP-1D (Syp), RhoA, Rac1, Grb2, Raf1, MEK kinase, MEK1, ERK1, ERK2, JNK1 (Santa Cruz Biotechnology Santa Cruz, CA); α-actinin (ICN Biomedicals, Costa Mesa, CA); Ras and GAP (Upstate Biotechnology, Lake Placid, NY); PI 3-kinase and Sos (Transduction Laboratories, Lexington, KY). A c-Yes polyclonal antibody also displayed a single band by Western immunoblotting using goat anti-mouse IgG conjugated with peroxidase (Amersham Corp., Arlington Heights, IL). Monoclonal antibodies also confirmed as monospecific by Western immunoblotting using goat anti-mouse IgG conjugated with peroxidase (Amersham Corp.) were paxillin, tensin, pp120, and protein kinase A (Transduction Laboratories); filamin (Soretoc, Indianapolis, IN); cortactin (Upstate Biotechnology); α-tubulin and LDH (Sigma); and vinculin (Upstate Biotechnology). c-Fak monoclonal antibody from Transduction Laboratories showed a single band using goat anti-mouse IgG conjugated with peroxidase (Pierce). Rhodamine-labeled phalloidin was from Molecular Probes (Eugene, OR). Integrin aggregation was determined for each ligand using rabbit antibodies 4318 and 4080 directed against the α5 and β1 integrin cytoplasmic domains, respectively (Larjava et al., 1990; LaFlamme et al., 1992; Miyamoto et al., 1995). Monospecific rabbit anti-talin antiserum was kindly provided by Dr. Keizo Takenaga, and mouse monoclonal VII F9 B1 anti-human vinculin by Dr. Victor Kotelskiy. FITC- and rhodamine-labeled secondary antibodies were obtained from BioSource International (Camarillo, CA).

**Use of Inhibitors**

Dose response curves were evaluated for selective tyrosine kinase inhibitors including the anti-Src agents genistein and herbimycin A and anti-EGF receptor inhibitors tyrphostin A23, tyrphostin A25, and erbstatin analog (Calbiochem Novabiochem Corp., San Diego, CA). Cells were cultured with each tyrosine kinase inhibitor at various concentrations for 24 h, and then incubated in medium with fibronectin-depleted serum and 25 μg/ml cycloheximide for 2 h as for other beads assays in the continual presence of inhibitor, and then suspended by trypsinization. When using genistein and herbimycin A, cells required 2 h rather than the usual 1 h to spread fully on the collagen-coated coverslips before the addition of beads. To monitor the cytoxicity of agents, total cell number was determined with a Coulter counter after 24 h incubation. Cytochalasin D was purchased from Calbiochem; for assays, cytochalasin D and beads were added simultaneously to cells on collagen-coated coverslips and assayed as described (Miyamoto et al., 1995).

**Extraction of Total Cell Protein and Proteins Bound to Beads**

Cells were incubated with fibronectin depleted serum and 25 μg/ml cycloheximide, as described above. To assess the effect of each pharmacological agent on protein tyrosine phosphorylation, cells were rinsed with serum-free medium and incubated without serum for 1 h, and then cells (5 × 10⁶ in 1 ml serum-free medium) were incubated with 10⁷ beads for 20 min at 37°C. The cells were rinsed with PBS containing 1 mM sodium orthovanadate, and then lysed with RIPA buffer (1% Triton X-100, 1% SDS, 150 mM NaCl, 50 mM Tris, pH 8.0, 0.2 U/ml aprotinin, 2 μg/ml leupeptin, 1 μg/ml pepstatin A, 2 μM PMSF, 1 mM sodium orthovanadate). To identify the proteins bound to beads, cells were suspended (10⁷ cells/ml) in serum-free medium with the beads (~2 × 10⁷) and rotated gently for 20 min at 37°C. The complex of beads and cells was then washed with modified CSK extraction buffer (0.5% Triton X-100, 50 mM NaCl, 300 mM sucrose, 3 mM MgCl₂, 3 mM MnCl₂, 0.2 U/ml aprotinin, 2 μg/ml leupeptin, 1 μg/ml pepstatin A, 2 μM PMSF, 10 mM Pipes [1,4-piperazineethanesulfonic acid], 40 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mM sodium orthovanadate) without detergent at 4°C. The pellet containing beads and cells was suspended with CSK buffer, and then

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**Figure 1.** Integrin-induced transmembrane aggregation of Src kinase family members, Src substrates, and signaling molecules by individual ligand-coated beads. A. Immunofluorescence for juxtamembrane accumulation of tyrosine-phosphorylated proteins, c-Src, and c-Lyn induced by beads coated with fibronectin or polylysine. Each inset shows a higher magnification view, focusing on the equator of the bead marked by a highlighted black arrowhead; white arrowhead indicates a second bead bound to the cell. Fibronectin-coated beads induce localization of tyrosine-phosphorylated proteins after 20 min (a). The same cell is shown in the panel below by combined transmitted light and fluorescence to illuminate the bead (c). Immunolocalization of c-Src protein is also found adjacent to fibronectin-coated beads (e). Lack of tyrosine-phosphorylated protein adjacent to polylysine-coated beads is observed (b), plus transmitted light to illuminate the bead (d): absence of c-Src immunolocalization (f). No immunostaining of c-Lyn protein adjacent to either fibronectin- or polylysine-coated beads is observed (g and h).

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Figure 1. (B) Quantitation of bead-induced accumulation of Src family kinases, Src substrates, and signaling molecules with an SH2 domain as assessed by immunofluorescence microscopy. The ordinate indicates the percentage of beads positive for immunofluorescence for each antigen listed on the abscissa; values indicate mean and standard deviation. Polystyrene beads were coated with one of the following ligands: fibronectin, GRGDSPC peptide disulfide-conjugated to IgG (multivalent immobilized RGD ligand) according to the methods of Humphries et al. (1987), adhesion-blocking antibodies mAb 16 to the integrin α5 subunit and mAb 13 to the β1 subunit, non-inhibitory antibodies mAb 11 to the α3 subunit and mAb K20 to the β3 subunit, or Con A and polylysine as control beads. Bar, (A) 20 μm.

Assessment of Serine/Threonine Kinase Activation

Cells were preincubated with fibronectin-depleted serum with or without 25 μg/ml cycloheximide, detached with trypsin-EDTA, washed with serum-free medium, and incubated in serum-free medium for 1 h. Cells (2 × 10⁶) in 500 μl were incubated with 4 × 10⁷ beads at 37°C. For measurement of ERK activity, cells were extracted with 1% NP-40, 20 mM Heps, pH 7.5, 10 mM EGTA, 40 mM β-glycerophosphate, 2 mM Na₃VO₄, 1 mM DTT, 2.5 mM MgCl₂, 20 μg/ml aprotinin, 20 μg/ml leupeptin, 1 mM PMSF. Both ERK1 and ERK2 protein were immunoprecipitated at 4°C using anti-ERK1 or anti-ERK2 antibody (Santa Cruz Biotechnology), which has a cross reactivity to both ERK1 and ERK2 protein, and then with protein G Sepharose for 1 h. To analyze the activation of SAPK/JNK, cells were extracted with 0.1% Triton, 300 mM NaCl, 25 mM Heps, pH 7.5, 0.2 mM EDTA, 20 mM β-glycerophosphate, 1 mM Na₃VO₄, 1.5
**Results**

**Experimentally Induced Aggregation of Members of the Src Kinase Family, Src Substrates, and Signaling Molecules with SH2 Domains at Cell-Matrix Contact Sites**

Beads coated with extracellular matrix ligands such as fibronectin can mimic adhesion site generation, and they can induce transmembrane aggregation of a variety of cytoskeletal molecules. This rapid, massive accumulation of proteins associated with the actin cytoskeleton requires that integrin receptors be both aggregated and occupied by ligand (Miyamoto et al., 1995). To explore whether sig-
Figure 3. Effects on the aggregation of specific cytoskeletal proteins and signal transduction molecules of herbimycin A, genistein, or cytochalasin D. (A) Immunofluorescence for juxtamembrane accumulation of tyrosine-phosphorylated proteins, F-actin, and tensin induced by beads coated with fibronectin. Each inset shows a higher magnification view, focusing on the equator of the bead marked by a highlighted black arrowhead; white arrowhead indicates a second bead bound to the cell. Localization of tyrosine-phosphorylated proteins (a–c) and F-actin (d–f) becomes undetectable after treatment with each of these three agents when assayed using fibronectin-coated beads. In contrast, tensin accumulation is unaffected (g–i). (B) Quantitation of bead-induced accumulation of cytoskeletal and signaling molecules with or without inhibitors. The ordinate indicates the percentage of beads positive for immunofluorescence accumulation of each antigen listed on the abscissa; values indicate mean and standard deviation. Polystyrene beads were coated with each of the following ligands: fibronectin, GRGDSPC peptide disulfide conjugated to IgG, adhesion-blocking mAb 16 to α5 subunit and mAb 13 to β1 subunit, or noninhibitory mAb 11 to the α5 subunit and mAb K20 to the β1 subunit. Bar: (A) 20 μm.

Signal transduction molecules can also be physically redistributed into these localized sites by a transmembrane process, whether they can be functionally activated with specific time courses, and what triggering mechanisms might be involved, we examined a number of proteins involved in signaling pathways involving tyrosine phosphorylation. Tyrosine-phosphorylated proteins were recruited to contact sites by beads coated with fibronectin as expected (Fig. 1 A). Multivalent ligands and both adhesion-blocking and noninhibitory antibodies coated on beads also effectively induced transmembrane aggregation of tyrosine-phosphorylated proteins (Fig. 1 B).

Antibodies against specific signaling proteins were used to classify the types of molecule that accumulated. The tyrosine kinases FAK, c-Src, c-Fyn, c-Yes, and c-Csk were recruited to large adhesion complexes within 20 min according to immunofluorescence microscopy after induction of complexes by fibronectin, multivalent ligands, adhesion-blocking antibody, or even noninhibitory antibody immobilized on beads (Fig. 1, A and B). However, no significant clustering of the kinase c-Lyn could be observed with any type of bead tested (Fig. 1, A and B).

FAK and tensin appear to be particularly proximally recruited to these contact sites (Miyamoto et al., 1995), and both are potential Src substrates (Schaller et al., 1993). The recruitment of two other Src substrates was therefore compared. Immunofluorescence with anti-p120 and cortactin antibodies revealed a similarly striking transmembrane clustering near ligand-coated beads. Accumulations were induced even by noninhibitory antibody-coated beads, which could induce integrin and target molecule aggregation even in the absence of ligand occupancy (Fig. 1 B).

In addition, other signaling molecules that contain an SH2 domain for interactions with tyrosine phosphorylated sites showed substantial aggregation at contact sites of beads, including those coated with noninhibitory antibodies (Fig. 1 B). Although noninhibitory antibodies on beads also induced focal accumulations, the extent of aggregation appeared quantitatively lower when compared to complexes triggered by fibronectin, multivalent ligands, or
adhesion-blocking antibodies coated on beads (Fig. 1B).

Our protein localization results obtained using immunofluorescence were confirmed for several proteins using a modification of the method of Plopper and Ingber (1993; see Materials and Methods), which provides a method to detect biochemically the proteins associated with beads in focal adhesion-like complexes. The cytoskeletal and signaling molecules vinculin, paxillin, c-Src, GAP, and PI 3-kinase were found to be significantly enriched in association with fibronectin-coated beads compared to polylysine-coated beads (data not shown).

Taken together, these findings indicate that Src kinase family members, Src substrates, and many other (but importantly not all) signaling molecules contribute to the protein complex that is experimentally induced at matrix contact sites. Unlike many cytoskeletal molecules, these molecules can accumulate under less stringent conditions: integrin receptor aggregation but not necessarily integrin ligand occupancy, although the latter may enhance the accumulation. These findings raised two major questions: (1) Does redistribution and local accumulation of signaling molecules depend on tyrosine phosphorylation, and are there comparable requirements for various cytoskeletal molecules? and (2) What are the consequences of the various types of integrin-induced complex formation on signal transduction function, specifically on the SAPK/JNK pathway compared to the previously described integrin stimulation of the ERK1 and ERK2 members of the MAP kinase pathway (Chen et al., 1994; Morino et al., 1994; Schlaepfer et al., 1994)?

**Effects of Inhibitors on Integrin-induced Signaling and Cytoskeletal Protein Complexes**

Selective inhibitors of kinase and cytoskeletal function provide important insights, but their effects can include nonspecific cytotoxic effects on cell growth, viability, and other processes (Uehara et al., 1986; Akiyama et al., 1987; Levitzki, 1990). To reduce the likelihood of artifacts, we quantitated the effects of a range of concentrations of various kinase inhibitors on integrin-mediated recruitment of cytoplasmic tyrosine-phosphorylated molecules into complexes, compared to \( \alpha_5 \beta_1 \) integrin clustering and cytotoxicity, as measured by inhibition of cell growth or cytolysis. Herbimycin A has been used as a specific inhibitor of Src family tyrosine kinases (Uehara et al., 1986), while genistein is a more broad-spectrum inhibitor of tyrosine kinases as well as Src tyrosine kinase family members (Akiyama et al., 1987). Erbstatin analog and tyrphostins A23 and A25 are inhibitors of the EGF (epidermal growth factor) receptor kinase-dependent pathway (Levitzki, 1990). Cytochalasin D has been used to inhibit the polymerization of actin monomers, and has been shown to block FAK phosphorylation (Shattil et al., 1994b).

Dose-response curves were obtained after incubation with agents for 24 h, comparing the aggregation of total tyrosine-phosphorylated proteins induced by fibronectin-coated beads with the clustering of \( \alpha_5 \) and \( \beta_1 \) integrin subunits. Cell number and cytotoxic effects visible by phase contrast microscopy were also monitored simultaneously. Maximal concentrations that showed minimal cytotoxic effects were chosen based on the cell count data and morphological observations in Fig. 2 and its legend: 900 nM herbimycin A, 140 \( \mu \)M genistein, or 300 nM cytochalasin D. Each inset shows a higher magnification view, focusing on the equator of the bead marked by a highlighted black arrowhead; white arrowhead indicates a second bead bound to the cell. Localization of ERK2 and JNK1 is detected adjacent to fibronectin-coated beads (a and b). Lack of ERK2 and JNK1 immunolocalization adjacent to fibronectin-coated beads observed after treatment with herbimycin A (c and d), genistein (e and f), or cytochalasin D (g and h).

**Figure 4.** Aggregation of signal transduction molecules induced by individual ligand-coated beads. (A) Immunofluorescence for juxtamembrane accumulation of ERK2 and JNK1 induced by beads coated with fibronectin comparing controls (no agent: described as untreated), 900 nM herbimycin A, 140 \( \mu \)M genistein, or 300 nM cytochalasin D. Each inset shows a higher magnification view, focusing on the equator of the bead marked by a highlighted black arrowhead; white arrowhead indicates a second bead bound to the cell. Localization of ERK2 and JNK1 is detected adjacent to fibronectin-coated beads (a and b). Lack of ERK2 and JNK1 immunolocalization adjacent to fibronectin-coated beads observed after treatment with herbimycin A (c and d), genistein (e and f), or cytochalasin D (g and h).
Figure 4. (B) Quantitation of bead-induced accumulation of the signaling molecules RhoA, Rac1, Grb2, Sos, Ras, Raf1, MEKK, MEK1, ERK1, ERK2, and JNK1 by immunofluorescence microscopy in controls (no agent: described as untreated) or cells treated with herbimycin A, genistein, or cytochalasin D. The ordinate indicates the percentage of beads positive for immunofluorescence for each antigen listed on the abscissa; values indicate mean and standard deviation. Polystyrene beads were coated with fibronectin, GRGDSPC peptide disulfide-conjugated to IgG, adhesion-blocking antibodies mAb 16 to the α5 subunit and mAb 13 to the β1 subunit, noninhibitory antibodies mAb 11 to the α5 subunit and mAb K20 to the β1 subunit, and Con A or polylysine as controls.
phosphorylated proteins, F-actin, and c-Src (Fig. 2 and data not shown). At a concentration of 300 nM cytochalasin D, the aggregation of α5 and β1 integrin was similar to controls, but the accumulation of tyrosine-phosphorylated proteins was inhibited (Fig. 2).

At the optimal concentrations derived above, inhibition of tyrosine phosphorylation of individual proteins by Western immunoblotting using antibody to phosphotyrosine was confirmed: strong inhibition of tyrosine phosphorylation was observed in cells treated with 900 nM herbimycin A and 140 μM genistein (lane 5), whereas tyrosine phosphorylation of major bands was not inhibited in the cells treated with 200 μM tyrphostin A23, 200 μM tyrphostin A25, and 2.5 μg/ml erbstatin analog; cytochalasin D treatment significantly inhibited total cellular tyrosine phosphorylation at concentrations above 300 nM (data not shown).

Selective Effects of Inhibitors on Aggregation of Cytoskeletal and Signal Transduction Molecules

Cytoskeletal proteins and signaling molecules could be separated into distinct classes depending on their response to tyrosine kinase inhibitors, cytochalasin D, and ligand occupancy. None of the tyrosine kinase inhibitors or cytochalasin D affected the accumulation of the cytoskeletal
protein (and Src substrate) tensin at sites of integrin clustering induced by bead substrates of fibronectin, multivalent ligands, adhesion-blocking antibodies, or noninhibitory antibodies (Fig. 3, A and B). Three more cytoskeletal proteins, talin, α-actinin, and vinculin, were similarly resistant to all of these inhibitors, but their accumulation was dependent on substrates that provided receptor occupancy: no accumulation of talin, α-actinin, and vinculin was detected after clustering of integrins with noninhibitory antibodies in untreated or treated cells with herbimycin A, genistein, or cytochalasin D (Miyamoto et al., 1995 and data not shown). Finally, a marked inhibition of accumulation of a class of three other cytoskeletal molecules was detected after treatment with herbimycin A, genistein, or cytochalasin D: F-actin, paxillin (also a Src substrate), and filamin did not accumulate after treatment with these agents, but there was no effect after treatment with tyrphostin A23, tyrphostin A25, or erbstatin analog (Fig. 3 B and data not shown).

Signal transduction molecules fell into three classes, with no differences due to ligand occupancy. Several molecules (α-tubulin, LDH, c-Lyn, and protein kinase A) did...
not show accumulation near beads even with a combination of receptor occupancy and aggregation (Fig. 1 B, Miyamoto et al., 1995, and data not shown). Twenty other signaling-related molecules accumulated in response to simple integrin clustering by noninhibitory antibodies. The aggregation of FAK was resistant to inhibition by all agents tested, i.e., even though genistein, herbimycin A, and cytochalasin D inhibited total tyrosine phosphorylation, FAK aggregation was unchanged. In striking contrast, each of these three agents markedly inhibited the accumulation of other Src substrates including p120 and cortactin, members of the Src kinase family including c-Src, c-Fyn, and c-Yes, and of other types of signaling molecules including GAP, PI 3-kinase, PLC-γ, c-Csk, and PTP-1D. Moreover, molecules implicated in the MAP kinase pathways, ERK1, ERK2, and JNK1, were also sensitive to inhibition (see following section). These results were obtained regardless of whether the experimental induction of aggregation was triggered by beads coated with fibronectin, multivalent RGD ligands, adhesion-blocking antibodies, or noninhibitory antibodies (Fig. 3 B). These immunolocalization results were further confirmed by directly analyzing the proteins that remained associated with beads-associated complexes could be detected of GAP, PI 3-kinase, ERK1, or ERK2, as determined by Western immunoblotting (data not shown).

To summarize this section, these results establish the existence of distinct hierarchies of independence or dependence upon tyrosine phosphorylation, actin microfilament integrity, and ligand occupancy for redistribution and focal juxtamembrane accumulation. The cytoskeletal protein tensin and the kinase FAK were unique in their need for only integrin receptor aggregation; talin, α-actinin, and vinculin required the addition of integrin ligand occupancy; in contrast, other cytoskeletal proteins (3 more) and signaling molecules (19 more) failed to accumulate after treatment with certain tyrosine kinase inhibitors or cytochalasin. In addition, the cytoskeletal protein tubulin and the signaling protein c-Lyn were not aggregated by integrin ligation under any conditions.

### Comparisons of Signaling Pathways: Physical Aggregation and Function

We next focused on a series of other proteins implicated in tyrosine kinase signaling cascades. Marked focal aggregation of RhoA, Rac1, Ras, Raf1, Grb2, Sos, MEK kinase (MEKK), MEK1, ERK1, ERK2, and JNK1 could be experimentally induced by any of the specific integrin-interaction molecules fibronectin, multivalent RGD ligand, adhesion-blocking antibodies, and noninhibitory antibodies, but not by concanavalin A or polylysine (Fig. 4, A and B). Aggregation of all 11 of these signal transduction molecules was inhibited by the tyrosine kinase inhibitors herbimycin A and genistein, as well as by cytochalasin D (Fig. 4 B). Treatment with cycloheximide alone can induce activation of the SAPK/JNK pathway (Cosso et al., 1995). In this study, cycloheximide itself also partially activated the SAPK/JNK pathway, but not the ERK pathway, in human foreskin fibroblasts (data not shown). Nevertheless, there was no quantitative difference in the aggregation of ERK1, ERK2, and JNK1 in the presence or absence of cycloheximide (data not shown).

To evaluate mechanisms of integrin activation of ERK and potentially of SAPK/JNK, assays specific for each kinase were performed to compare time courses, responses to each type of bead, and inhibition by herbimycin A, genistein, or cytochalasin D. Both classes of kinase were activated by integrin ligation by fibronectin in a temporally distinct fashion. ERK activation was maximal at 30 min and dissipated completely by 60 min (Fig. 5 B), whereas activity of SAPK/JNK reached a rapid peak within 10 min and gradually declined afterwards (Fig. 5 C). In contrast, enhanced total cellular tyrosine phosphorylation was detected from 10 min through 120 min (Fig. 5 A).

For comparisons of roles of aggregation and ligand occupancy, assays for ERK were performed at the peak time of 30 min, while SAPK/JNK was assayed after incubation for 10 min. No reproducible differences could be detected between activation by fibronectin, multivalent RGD ligand, adhesion-blocking antibodies, and noninhibitory antibodies; polylysine and concanavalin A were nonactivating (Fig. 5, D and E). Each of the agents that inhibited accumulation of these kinases, herbimycin A, and genistein, blocked the activation of both types of MAP kinase (ERK and SAPK/JNK) caused by binding to fibronectin-coated beads (Fig. 5, F and G). Cytochalasin D inhibited activation of the ERK

### Table I. Molecular Hierarchies of Transmembrane Redistribution of Cytoplasmic Proteins by Integrins

|          | A          | B          | C          | D          |
|----------|------------|------------|------------|------------|
| Cytoskeletal molecules | Talin | α-actinin | Vinculin | F-actin |
| Src substrates | Tensin | Tensin | Tensin | FAK |
| Src Kinase family | FAK | FAK | FAK | FAK |
| Signaling molecules | FAK | FAK | FAK | FAK |

| A | B | C | D |
|---|---|---|---|
| Integrin aggregation | Integrin aggregation plus integrin occupancy | Integrin aggregation plus tyrosine phosphorylation | Integrin aggregation plus integrin occupancy, tyrosine phosphorylation, and actin cytoskeletal integrity. |

A, Integrin aggregation; B, Integrin aggregation plus integrin occupancy; C, Integrin aggregation plus tyrosine phosphorylation; D, Integrin aggregation plus integrin occupancy, tyrosine phosphorylation, and actin cytoskeletal integrity. Not redistributed: α-Tubulin, c-Lyn, PKA, LDH.
pathway, but did not block activation of the SAPK/JNK1 pathway (Fig. 5, F and G). However, the addition of cytochalasin D alone stimulated the SAPK/JNK pathway even without the addition of fibronectin-coated beads, but cytochalasin D did not activate the ERK pathway (data not shown), suggesting further differences between these pathways. Thus, integrins can mediate the activation of two distinct serine/threonine kinase pathways with different time courses and different responses to disruption of the actin cytoskeleton.

**Discussion**

Our major new conclusions from these studies on mechanisms of integrin transmembrane action are as follows: (a) Aggregation of integrin receptors, even in the absence of ligand occupancy, is sufficient to induce a prompt transmembrane accumulation of a large class of at least 20 signal transduction molecules, including c-Src, c-Fyn, RhoA, Rac1, Ras, GAP, MEK1, ERK1, ERK2, and JNK1. In contrast, c-Lyn was not responsive to integrin clustering. (b) Integrin aggregation with or without ligand occupancy also triggers activation of both ERK and SAPK/JNK signal transduction pathways, but with different time courses of maximal stimulation and different responses to cytochalasin D, suggesting distinct mechanisms. (c) The accumulation of most signal transduction molecules as well as activation of both ERK and SAPK/JNK signal transduction pathways, but with different time courses of maximal stimulation and different responses to cytochalasin D, suggesting distinct mechanisms. (c) The accumulation of most signal transduction molecules as well as activation of both ERK and SAPK/JNK signal transduction pathways, but with different time courses of maximal stimulation and different responses to cytochalasin D, suggesting distinct mechanisms. (c) The accumulation of most signal transduction molecules as well as activation of both ERK and SAPK/JNK signal transduction pathways, but with different time courses of maximal stimulation and different responses to cytochalasin D, suggesting distinct mechanisms. (d) Tyrosine kinase-mediated phosphorylation and actin cytoskeletal integrity, as well as integrin occupancy and aggregation, are required for integrin-induced accumulation of one group of three cytoskeletal components: F-actin, paxillin, and filamin. (e) In contrast, integrin-induced aggregation of a second group of four cytoskeletal proteins comprised of tensin, talin, α-actinin, and vinculin can proceed even after inhibition of the tyrosine phosphorylation necessary for accumulation of other signaling and cytoskeletal molecules. (f) Talin, α-actinin, and vinculin comprise a unique subset of three cytoskeletal proteins whose membrane accumulation requires both integrin aggregation and occupancy, but not the tyrosine kinase activity needed for many other integrin-responsive proteins.

The binding of ligand-coated beads provides a rapid method for experimentally triggering strong transmembrane adhesive complexes based on receptor contacts with extracellular matrix that mimic focal and matrix adhesions (Mueller et al., 1989; Plopper and Ingber, 1993; Lewis and Schwartz, 1995). This process can be distinguished from phagocytosis by a strong juxtacellular membrane accumulation of myosin II (unpublished data), compared to the absence of myosin II adjacent to classical phagocytic vesicles (Brown, 1995). The findings in this study, when combined with the previously established function of ligand occupancy alone in fibroblasts (LaFlamme et al., 1992 and references therein), allow us to distinguish a minimum of at least six distinct molecular classes of transmembrane response to cell surface integrin interactions. Besides unique responses to integrin occupancy alone and its synergy with aggregation, Fig. 6 shows three additional states; some molecules have no response to any of these stimuli.

Although not contradictory to previous studies, these classifications based on functional responses to integrin interactions identify novel categorical groupings of over 30 cytoskeletal and signaling molecules (Table I). FAK was the only tyrosine kinase out of five tested (c-Src, c-Fyn, c-Yes, c-Csk, and FAK) that could associate with integrin clusters upon inhibitory tyrosine phosphorylation by genistein or herbimycin A. This kinase can bind to β1 integrin cytoplasmic tails in vitro (Schaller and Parsons, 1994), and this association appears more proximal than the two other molecules known to bind to this integrin intracellular domain, i.e., talin and α-actinin (Horwitz et al., 1986; Otey et al., 1990; but see also Lewis and Schwartz, 1995). Although this central positioning of FAK suggests an important early role in fibroblast integrin responses, platelet FAK phosphorylation appears to be relatively late in the sequence of phosphorylation events that occur during platelet activation (e.g., compare Schaller et al., 1992 with Huang et al., 1993).

Even though tensin is a tyrosine kinase substrate like paxillin, pp120, and cortactin (Schaller et al., 1993), it has unique characteristics in integrin-mediated localization studies. No other tyrosine kinase substrate (except FAK itself) or cytoskeletal protein shows this resistance to inhibition of tyrosine phosphorylation or disruption of actin cytoskeletal integrity.

Our previous study demonstrated that talin, α-actinin, paxillin, F-actin, and other cytoskeletal molecules require both integrin aggregation and ligand occupancy for accumulation (Miyamoto et al., 1995). The present study now identifies two distinct classes among these molecules. Talin, α-actinin, and vinculin comprise a unique group of molecules that is able to accumulate focally even if tyrosine phosphorylation is inhibited by genistein or herbimycin A, and even if actin-related processes are disrupted by cytochalasin D. Since talin and α-actinin have the capacity to interact with β1 integrin cytoplasmic domains (Horwitz et al., 1986; Otey et al., 1990; Lewis and Schwartz, 1995), it is possible that the three molecules can accumulate in direct association with integrins once the requirements of clustering and occupancy are fulfilled; vinculin might associate via binding to α-actinin or talin (e.g., see Burridge et al., 1988). Tyrosine phosphorylation inhibitable by genistein and herbimycin A would, thus, not be involved in this process.

Paxillin, F-actin, and filamin form another distinct group of cytoskeletal molecules that require all three types of input, i.e., tyrosine kinase-mediated functions in addition to integrin aggregation and occupancy. The biologically important association of F-actin with integrins is therefore likely to be subjected to maximal regulatory modulation because of this mandatory tripartite input. Paxillin can be tyrosine phosphorylated via FAK, and has binding sites for vinculin, c-Src, v-Crk, and c-Csk (e.g., see Clark and Brugge, 1995). It is possible that the aggregation of paxillin provides a mechanism for regulating cytoskeletal organization, accompanied by aggregation of signaling molecules.
Physical focal submembrane accumulation of signaling molecules required integrin aggregation, but not occupancy. This pattern was consistent with that of FAK and tensin. However, in marked contrast to these two molecules, the accumulation process was blocked by the inhibitors of Src kinases, genistein and herbimycin A, but not by other tyrosine kinase inhibitors. Puzzlingly, disruption of actin cytoskeletal integrity by cytochalasin D also disrupted this accumulation, as has been observed in platelets (Shattil et al., 1994b). The effect is accompanied by inhibition of overall tyrosine phosphorylation detected by Western blotting, suggesting a role for tyrosine phosphorylation in the mechanism. It is important to note that this effect was found even under conditions where accumulations of actin were not present (which requires an additional ligand occupancy step). Cells have ubiquitous F-actin filaments in the cell cortex near where integrin-ligand occupancy step). Cells have ubiquitous F-actin filaments in the cell cortex near where integrin-cyto-
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