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29. We thank all those individuals who have contributed to the Portal Project over the past 17 years. Supported by NSF, most recently with grant DEB-9221238.

5 July 1994; accepted 29 November 1994

Synergistic Roles for Receptor Occupancy and Aggregation in Integrin Transmembrane Function

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Integrin receptors mediate cell adhesion, signal transduction, and cytoskeletal organization. How a single transmembrane receptor can fulfill multiple functions was clarified by comparing roles of receptor occupancy and aggregation. Integrin occupancy by monovalent ligand induced receptor redistribution, but minimal tyrosine phosphorylation signaling or cytoskeletal protein redistribution. Aggregation of integrins by noninhibitory monoclonal antibodies on beads induced intracellular accumulations of pp125^{FAK} and tensin, as well as phosphorylation, but no accumulation of other cytoskeletal proteins such as talin. Combining antibody-mediated clustering with monovalent ligand occupancy induced accumulation of seven cytoskeletal proteins, including α-actinin, talin, and F-actin, thereby mimicking multivalent interactions with fibronectin or polyvalent peptides. Integrins therefore mediate a complex repertoire of functions through the distinct effects of receptor aggregation, receptor occupancy, or both together.

Transmembrane integrin receptors for extracellular matrix proteins mediate signal transduction, but they also participate in organizing cell adhesion sites and the actin-containing cytoskeleton (*1*). How a single receptor can fulfill these multiple functions, as well as show selectivity in effects on specific cytoskeletal proteins, was examined by comparing the relative roles of receptor occupancy and aggregation. We hypothesized that even though simple aggregation of a number of cytokine and hormone receptors can mediate normal transmembrane signal transduction in place of ligand occupancy (*2*), some receptors might be able to assign distinct functions to three types of transmembrane signal: ligand occupancy, receptor aggregation, or both together. We determined the requirements for integrin-mediated transmembrane control of the localization of specific cytoskeletal proteins and for transmembrane signaling involving tyrosine phosphorylation. We found that (i) integrin receptors induce distinct cellular responses to binding of a ligand, to aggregation, or to a combination of the two; (ii) integrin receptors can control the distribution of specific cytoskeletal proteins (one protein was controlled by simple receptor aggregation, whereas six other cytoskeletal proteins required a combination of both aggregation and ligand binding); and (iii) the most proximal molecular interactions with integrin cytoplasmic domains appear to involve the cytoskeletal protein tensin and the tyrosine kinase pp125^{FAK} (focal adhesion kinase), rather than the previous candidates talin and α-actinin.

Polystyrene beads coated with substrates and antibodies served as mediators of integrin occupancy or aggregation, whereas soluble monovalent ligands provided simple ligand occupancy. Beads coated with fibronectin mimic adhesive site generation (*3*) and can induce transmembrane aggregation of a variety of cytoskeletal molecules including F-actin (Fig. 1, A and B). We developed procedures to quantitate this process and detected similar bead-induced transmembrane aggregation with talin, α-actinin, tensin, vinculin, paxillin, and filamin (Fig. 2A) (*4*, *5*). In addition, pp125^{FAK} also showed bead-induced clustering, whereas tubulin did not (Figs. 1C and 2A). Similar results were obtained with beads coated with multivalent Arg-Gly-Asp (RGD)–containing peptide conjugates (Fig. 2A) and with adhesion-blocking monoclonal antibodies (mAbs) to either the α_5 or β_1 subunit of the fibronectin receptor (Fig. 1, G to I, and Fig. 2B). These multivalent antibody ligands induced transmembrane aggregation of all seven cytoskeletal proteins and FAK, but not tubulin, lactate dehydrogenase, or Jak-2 (Fig. 1C) (*6*). Little or no clustering of any cytoskeletal protein or FAK was observed with beads coated with polylysine or concanavalin A (Fig. 1, D to F, and Fig. 2A).

Unexpectedly, mAbs that do not inhibit cell adhesion displayed a distinct and specific subset of these properties. The mAbs 11, to the α_5 subunit, and K20, to the β_1 subunit, do not inhibit cell adhesion (*6-9*) and will hereafter be termed noninhibitory antibodies. Each of these noninhibitory mAbs coated on beads effectively induced transmembrane aggregation of tensin in patterns that were indistinguishable from tensin aggregation induced by adhesion-blocking mAbs and multivalent ligands (Figs. 1K and 2B). However, no significant coclustering of F-actin, talin, α-actinin, vinculin, paxillin, or filamin could be detected (Figs. 1J and 2B). Interestingly, FAK was also readily coclustered by these noninhibitory mAbs (Figs. 1L and 2B). Three other mAbs to the β_1 subunit were compared and had similar patterns; for example, noninhibitory mAbs DF5 and LM534 induced aggregation of FAK but not F-actin, whereas adhesion-blocking mAb P4C10 induced aggregation of both FAK and F-actin (*6*).

It is now well established that ligand- or antibody-induced clustering of a number of growth factor, hormone, and other receptors can mediate transmembrane signal transduction, including integrin-mediated tyrosine phosphorylation of FAK (*2*, *10*). As expected if simple aggregation alone triggers integrin signaling, beads coated with noninhibitory mAbs that do not bind to an active site stimulated tyrosine phosphorylation that could be detected by anti-

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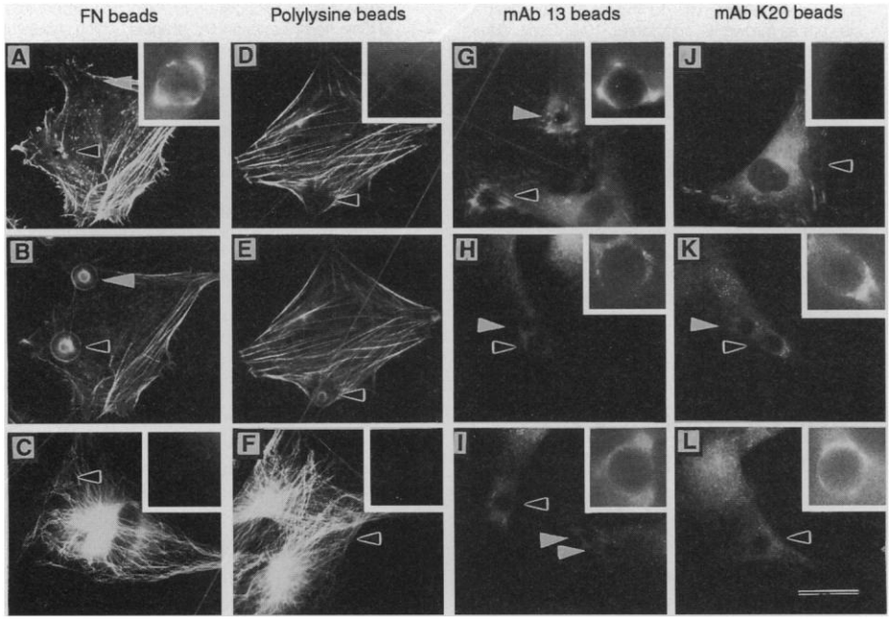

Fig. 1. Immunofluorescence for juxtamembrane accumulation of cytoskeletal proteins and FAK induced by beads coated with ligands or antibodies to integrin (*4*). Each inset shows a higher-magnification view, focusing on the equator of the bead marked by a highlighted black arrowhead; white arrowhead indicates a second bead bound to the cell. (**A** to **C**) Fibronectin-coated beads. Localization of F-actin as detected by rhodamine-labeled phalloidin (A), compared with combined transmitted light and fluorescence to illuminate the bead (B); no tubulin immunolocalization was detected adjacent to these beads (C). Lack of F-actin adjacent to polylysine-coated beads (**D**), and transmitted light to illuminate the bead (**E**); absence of tubulin immunolocalization (**F**). Effects of beads coated with adhesion-blocking mAb 13 (**G** to **I**) or noninhibitory mAb K20 (**J** to **L**) (both mAbs bind the β_1 subunit) on immununolocalization of paxillin (G and J), tensin (H and K), and FAK (I and L). Scale bar, 20 μm.

bodies to phosphotyrosine, as was observed with fibronectin and adhesion-blocking antibodies (Fig. 3) (*11*). Little or no tyrosine phosphorylation was detected after binding of beads coated with polylysine, even after the addition of high doses of soluble Gly-Arg-Gly-Asp-Ser (GRGDS) peptide to induce ligand occupancy (Fig. 3). Similarly, little or no signaling was observed in cells cultured on polylysine substrates and incubated with GRGDS peptide or a cell-binding fibronectin peptide of 75 kD at concentrations that readily blocked cell adhesion to fibronectin (6); under these conditions, α_5 and β_1 integrins were redistributed to preexisting focal contacts as described (*7*). Although these experiments confirm that clustering of integrin receptors can induce tyrosine phosphorylation, they also demonstrate that direct ligand occupancy by monovalent ligand is not a sufficient signal.

To explore further the relation between cytoskeletal organization and ligand occupancy, we examined the clustering of cytoskeletal proteins using beads coated with noninhibitory mAbs in the presence or absence of soluble ligand to induce receptor occupancy. Beads coated with noninhibitory mAbs induced aggregation of the remaining six actin-associated cytoskeletal proteins if the bead-clustered integrins were also occupied by GRGDS peptide (Fig. 2, C and D). Substituting the routine control GRGES

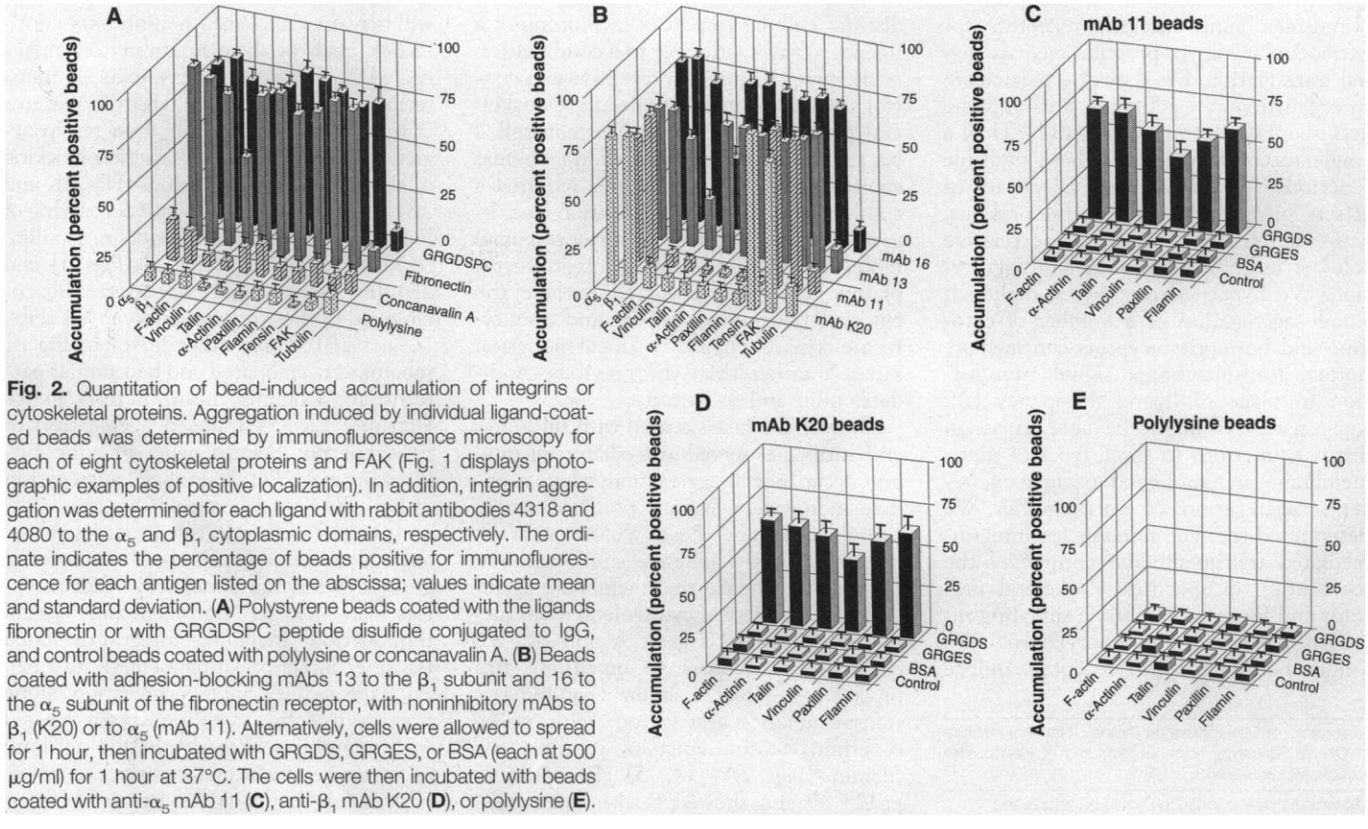

Fig. 2. Quantitation of bead-induced accumulation of integrins or cytoskeletal proteins. Aggregation induced by individual ligand-coated beads was determined by immunofluorescence microscopy for each of eight cytoskeletal proteins and FAK (Fig. 1 displays photographic examples of positive localization). In addition, integrin aggregation was determined for each ligand with rabbit antibodies 4318 and 4080 to the α_5 and β_1 cytoplasmic domains, respectively. The ordinate indicates the percentage of beads positive for immunofluorescence for each antigen listed on the abscissa; values indicate mean and standard deviation. (**A**) Polystyrene beads coated with the ligands fibronectin or with GRGDSPC peptide disulfide conjugated to IgG, and control beads coated with polylysine or concanavalin A. (**B**) Beads coated with adhesion-blocking mAbs 13 to the β_1 subunit and 16 to the α_5 subunit of the fibronectin receptor, with noninhibitory mAbs to β_1 (K20) or to α_5 (mAb 11). Alternatively, cells were allowed to spread for 1 hour, then incubated with GRGDS, GRGES, or BSA (each at 500 μg/ml) for 1 hour at 37°C. The cells were then incubated with beads coated with anti-α_5 mAb 11 (**C**), anti-β_1 mAb K20 (**D**), or polylysine (**E**).

peptide (Glu substituted for Asp) resulted in no enhanced cytoskeletal protein aggregation, confirming specificity (Fig. 2, C and D). As noted above, no clustering of cytoskeletal proteins occurred adjacent to polylysine-coated beads, even if integrin receptors were occupied with high concentrations of the GRGDS peptide ligand (Fig. 2E). These soluble RGD-containing peptides and fragments of fibronectin have been characterized for their binding parameters and effects on cell adhesion (*12*), which were confirmed for the cells used in this study by similar adhesive competitive inhibition studies (*6*). The RGD-containing peptides interact with fibroblastic cells with an inhibition constant (K_i) of ~100 μM (*12*); at the concentrations used in this study, at least 85 to 90% of the receptors should be occupied.

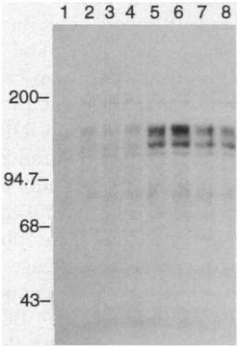

Fig. 3. Effects of beads coated with different ligands on protein tyrosine phosphorylation in human foreskin fibroblasts. Cells were incubated in the absence of beads and ligand (lane 1), in the presence of uncoated beads (lane 2), in the presence of polylysine-coated beads with (lane 3) or without (lane 4) GRGDS peptide (500 μg/ml), with anti-β_1 mAb K20–coated beads (lane 5), anti-β_1 mAb 13–coated beads (lane 6), anti-α_5 mAb 16–coated beads (lane 7), or fibronectin-coated beads (lane 8). Molecular sizes are indicated in kilodaltons.

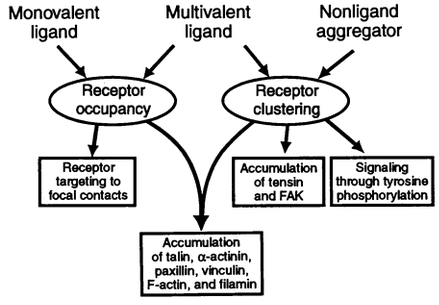

Fig. 4. Diagram summarizing proposed roles of receptor occupancy and receptor clustering in integrin responses to extracellular ligands. Unlike soluble growth factors and hormones, extracellular matrix molecules often exist as multivalent ligands immobilized in matrices or fibrils (*16*). Their functions in inducing ligand occupancy and formation of receptor clustering can be separated experimentally by the use of the monovalent peptide ligand GRGDS or beads coated with noninhibitory antibodies to integrin (nonligand aggregator), respectively. Each activating stimulus produces specific effects, and their actions synergize to induce accumulation of most cytoskeletal proteins examined. Extracellular matrix aggregates or matrix-coated beads produce all sets of responses because they induce both ligand occupancy and receptor clustering as a result of their multivalency.

We conclude that most integrin transmembrane effects on cytoskeletal protein organization require both ligand occupancy and the clustering of integrin receptors. The notable exceptions to this generalization are the cytoskeletal protein tensin and the FAK kinase, which can be aggregated in the absence of ligand occupancy. This finding suggests that the interactions of these two molecules are more proximal in the cascade of interactions with integrin receptors than even the known β_1 integrin-binding proteins talin (*13*) and α-actinin (*14*). In addition, the local accumulation of FAK may promote tyrosine phosphorylation.

The separation of integrin-fibronectin receptor functions involving ligand occupancy from those involving aggregation provides regulatory mechanisms ideally suited to integrin cell-adhesive and signaling functions (Fig. 4). Occupancy by even a monovalent ligand such as a proteolytic fragment induces receptor redistribution to preexisting adhesion sites (*7*). Initial integrin aggregation, even if ligand binding is lost, leads to a clustering of tensin and FAK with concomitant signaling involving tyrosine phosphorylation. Intracellular organization of large cytoskeletal complexes mimicking those found in focal contacts (*15*), however, appears to require both aggregation and occupancy of integrins. Extracellular matrix molecules are often multivalent as a result of adsorption to substrates or organization into fibrils (*16*), providing a direct mechanism for inducing adjacent integrin aggregation. Large, immobile accumulations of integrins and cytoskeletal molecules often accompany reduced cell migration rates (*17*). Regulation of integrin functions by these three mechanisms can allow a single integrin transmembrane receptor to function selectively in translocation, signaling, or different cytoskeleton-binding functions depending on the local environmental inputs.

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5. Induction of aggregation was calculated as the ratio *A/B*, where *A* equals the number of beads with circumferential immunolocalization and *B* equals the total number of beads; only beads whose circumference was located entirely within the outline of cells were counted. For each condition, at least 50 beads ($B = 50$) were scored per experimental condition, then each experiment was repeated five more times, and means and standard deviations were calculated for the sum of all six experiments ($n = 6$). Reagents: rhodamine-labeled phalloidin (Molecular Probes); antibodies to α-actinin (ICN); paxillin, tensin, and FAK (Transduction Laboratories, Lexington, KY); filamin (Serotec); and α-tubulin (Sigma). Rabbit antiserum to talin was provided by K. Takenaga and mAb VII F9 B11 to human vinculin by V. Koteliansky.
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20. We thank A. Utani, B. Z. Katz, J. P. Thiery, and M. Takeichi for many helpful discussions.

8 September 1994; accepted 6 December 1994