

Integrins Can Collaborate with Growth Factors for Phosphorylation of Receptor Tyrosine Kinases and MAP Kinase Activation: Roles of Integrin Aggregation and Occupancy of Receptors

Shingo Miyamoto, Hidemi Teramoto,* J. Silvio Gutkind,* and Kenneth M. Yamada

Laboratory of Developmental Biology and *Laboratory of Cellular Development and Oncology, National Institute of Dental Research, National Institutes of Health, Bethesda, Maryland 20892-4370

Abstract. Integrins mediate cell adhesion, migration, and a variety of signal transduction events. These integrin actions can overlap or even synergize with those of growth factors. We examined for mechanisms of collaboration or synergy between integrins and growth factors involving MAP kinases, which regulate many cellular functions. In cooperation with integrins, the growth factors EGF, PDGF-BB, and basic FGF each produced a marked, transient activation of the ERK (extracellular signal-regulated kinase) class of MAP kinase, but only if the integrins were both aggregated and occupied by ligand. Transmembrane accumulation of total tyrosine-phosphorylated proteins, as well as nonsynergistic MAP kinase activation, could be induced by simple integrin aggregation, whereas enhanced transient accumulation of the EGF-receptor substrate eps8 required

integrin aggregation and occupancy, as well as EGF treatment. Each type of growth factor receptor was itself induced to aggregate transiently by integrin ligand-coated beads in a process requiring both aggregation and occupancy of integrin receptors, but not the presence of growth factor ligand. Synergism was also observed between integrins and growth factors for triggering tyrosine phosphorylation of EGF, PDGF, and FGF receptors. This collaborative response also required both integrin aggregation and occupancy. These studies identify mechanisms in the signal transduction response to integrins and growth factors that require various combinations of integrin aggregation and ligands for integrin or growth factor receptors, providing opportunities for collaboration between these major regulatory systems.

INTEGRINS mediate a wide variety of biological processes by functioning as receptors and transmembrane transducers in cell adhesion, migration, and signal transduction events (Hynes, 1992; Gumbiner, 1993; Clark and Brugge, 1995; Schwartz et al., 1995; Yamada and Miyamoto, 1995; Rosales et al., 1995; Richardson and Parsons, 1995; Parsons, 1996; Ruoslahti, 1996; Gumbiner, 1996). For example, interactions of ligands with integrins can stimulate a variety of signaling events including tyrosine phosphorylation (reviewed by Schwartz et al., 1995; Clark and Brugge, 1995; Richardson and Parsons, 1995), cytoplasmic alkalization (Schwartz, 1993), calcium influx (Tucker et al., 1990; Schwartz, 1993; Schwartz and Denninghoff, 1994), activation of two types of mitogen-activated protein (MAP)¹ kinases, i.e., extracellular signal-

related kinase (ERK) (Chen et al., 1994; Schlaepfer et al., 1994; Morino et al., 1995; Zhu and Assoian, 1995) and c-Jun NH₂-terminal kinase or JNK (Miyamoto et al., 1995b), accumulation of cytoskeletal molecules at sites of cell adhesion to extracellular matrix (reviewed by Turner and Burridge, 1991; Sastry and Horwitz, 1993; Geiger et al., 1995; Yamada and Miyamoto, 1995), and altered gene expression (Damsky and Werb, 1992; Miyake et al., 1993; Lin et al., 1994; Fan et al., 1995; Delcommenne and Streuli, 1995; Mondal et al., 1995; Lafrenie and Yamada, 1996). Both the occupancy of integrin receptors by a ligand and integrin clustering into aggregates play roles in integrin function, and these two stimuli can synergize (Yamada and Miyamoto, 1995). Ligand-mediated integrin clustering has also been reported to induce the aggregation of the growth factor receptor for FGF (Plopper et al., 1995), and to stimulate the phosphorylation of PDGF β -receptors even in the absence of any growth factor ligand (Sundberg and Rubin, 1996).

Integrins can collaborate or synergize functionally with growth factors in a variety of biological processes (reviews include Damsky and Werb, 1992 and Schwartz et al., 1995). Some examples include cell growth and/or differentiation

Please address all correspondence to K. Yamada, Laboratory of Developmental Biology, NIDR, NIH, Building 30, Room 421, 30 Convent Drive MSC 4370, Bethesda, MD 20892-4370. Tel.: (301) 496-9124. Fax: (301) 402-0897. E-Mail: ky4w@nih.gov

1. *Abbreviations used in this paper:* bFGF, basic FGF; eps8, EGF-receptor substrate 8; ERK, extracellular signal-related kinase; MAP, mitogen-activated protein.

of fibroblasts, mammary epithelial cells, myoblasts, and chondrocytes (Blum et al., 1987; Tucker et al., 1990; Arner and Tortorella, 1995; Zhu and Assoian, 1995; Zhu et al., 1996; Sastry et al., 1996), and activation of the Na-H antiporter via a pathway dependent on protein kinase C (Schwartz and Lechene, 1992). Although increasing numbers of such examples of integrin synergism with growth factor pathways are being described at the biological level, the possible intermediates and mechanisms of such synergies are generally still unknown. An interesting exception is a mechanism involving dual effects on enzyme activation and levels of a substrate, thereby generating a synergistic response: integrins increase the activity of a PIP 5-kinase via the regulatory molecule Rho, generating the substrate 4,5-PIP₂, while PDGF concurrently activates the enzyme phospholipase C_γ, which hydrolyzes this substrate to generate second messengers (McNamee et al., 1993; Chong et al., 1994). In another system, the mitogenic effects of insulin are enhanced by interaction of vitronectin with α_vβ₃, which may be mediated via tyrosine phosphorylation of IRS-1, which binds to this integrin (Vuori and Ruoslahti, 1995).

Theoretically, a particularly direct mechanism of integrin-growth factor synergy might be for the downstream effects of integrins and of occupied growth factor receptors to be additive or synergistic. Since the MAP kinase pathway has been implicated as a regulator of such a wide variety of cell growth, differentiation, and gene activation pathways, and because integrins and growth factors can each separately stimulate this pathway, we have explored the possibility that integrins and growth factors might function cooperatively with respect to MAP kinase activation.

We have tested this hypothesis using human fibroblasts in suspension interacting with ligand-coated beads and soluble growth factors, avoiding conditions of long-term serum starvation. We find both a marked activation of ERK and upstream synergistic tyrosine phosphorylation of the cognate receptors following appropriate integrin stimulation of human fibroblasts in cooperation with EGF, PDGF, or basic FGF (bFGF). This process requires that the integrin receptors be both aggregated and occupied by ligand. This latter dual requirement mirrors requirements for both integrin aggregation and occupancy to induce the physical aggregation of each of these receptors at sites of integrin clustering. The assembly of integrins and growth factor receptors into these large signaling and cytoskeletal complexes, after matrix contact involving both integrin aggregation and occupancy, could explain the synergy we observe by transiently concentrating effectors and substrates together at a local site for greater mutual interaction.

Materials and Methods

Cell Culture and Bead Assays

Human foreskin fibroblasts were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum (Hyclone, Logan, CT), 1 mM glutamine, 50 μg/ml streptomycin, and 50 U/ml penicillin. The cells were a gift from Susan Yamada (National Institute of Dental Research, NIH) and were used at cell passages 9–18. Cells were maintained in regular medium, avoiding any serum starvation until after trypsinization.

Latex beads (mean diameter 11.9 μm, Sigma Chem. Co., St. Louis,

MO) were coated with ligand or antibody and incubated with cells as described previously for the experimental induction of focal integrin clustering and transmembrane accumulation of cytoplasmic molecules (Miyamoto et al., 1995a). Cells were routinely preincubated for 2 h in fibronectin-depleted serum with 25 μg/ml cycloheximide to prevent fibronectin secretion, detached with trypsin-EDTA, and then allowed to recover from the trypsinization in DMEM containing fibronectin-depleted serum for 20 min. The cells were then washed twice with serum-free medium and incubated in suspension in serum-free DMEM for an additional 30 min (total of ~40 min in serum-free medium). After a 20-min incubation with ligand- or antibody-coated beads in serum-free DMEM in suspension, cells were treated for 5 min at 37°C with or without the following growth factors from BioSource International (Camarillo, CA): 10 or 100 ng/ml human recombinant epidermal growth factor (EGF), 10 ng/ml human recombinant platelet-derived growth factor-BB (PDGF-BB), or 10 ng/ml human recombinant basic fibroblast growth factor (bFGF) for 5 min at 37°C. The peak of MAP kinase (ERK) activation after stimulation of human fibroblasts with these growth factors was determined in preliminary studies to occur routinely at this 5-min time point.

For immunofluorescence, 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., 1995a) using a Nikon HFX-II microscope equipped for fluorescein and rhodamine fluorescence. In experiments using mouse anti-β₁ K20 antibody on beads, an excess of rat monoclonal anti-mouse IgG_{2a} 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 16 to the α₅ integrin subunit and mAb 13 to the β₁ integrin subunit (Akiyama et al., 1989; LaFlamme et al., 1992), as well as mouse monoclonal antibody K20 to the β₁ integrin subunit (Immunotech, Westbrook, ME). Polyclonal rabbit anti-growth factor receptor antibodies against EGF-receptor, PDGF α-receptor, PDGF β-receptor, and bFGF-receptor were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal antibodies against FAK and epidermal growth factor substrate 8 (eps8) were from Transduction Laboratories (Lexington, KY) and against phosphotyrosine (mAb 4G10) were from Upstate Biotechnology Inc. (Lake Placid, NY). Sheep anti-mouse IgG conjugated with peroxidase (Amersham Corp., Arlington Heights, IL) was used as the secondary antibody for Western immunoblotting. FITC- and rhodamine-labeled secondary antibodies were obtained from BioSource International.

Characterization of Protein Tyrosine Phosphorylation

Cells were preincubated with DMEM with fibronectin-depleted serum and 25 μg/ml cycloheximide, detached with trypsin-EDTA, and allowed to recover for 20 min in fibronectin-depleted medium as described (LaFlamme et al., 1992). To assess the effect of each growth factor and integrins on protein tyrosine phosphorylation, cells were rinsed twice with serum-free DMEM and incubated for an additional 30 min without serum, and then 5 × 10⁵ cells in 500 μl serum-free medium were incubated with 10⁷ beads for 20 min at 37°C. The aggregated complexes of cells and beads were incubated with or without 10 ng/ml of each growth factor for an additional 5 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% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 50 mM Tris-Cl, pH 8.0, 0.2 U/ml aprotinin, 2 μg/ml leupeptin, 1 μg/ml pepstatin A, 2 mM PMSF, 1 mM sodium orthovanadate). The extracts were subjected to SDS-PAGE and Western immunoblotting using anti-phosphotyrosine antibody (Guan et al., 1991).

Measurement of Extracellular Signal-regulated Kinase Activation

Cells prepared as described above were incubated in suspension in serum-free DMEM for 30 min. Cells (10⁶) were then incubated with 2 × 10⁷ beads in 500 μl serum-free medium for 20 min at 37°C. The aggregated complexes of cells and beads were treated with or without each growth factor (10 ng/ml) for 5 min at 37°C. For measurement of ERK activity, cells were extracted with 1% NP-40, 20 mM Hepes, pH 7.5, 10 mM EGTA, 40 mM β-glycerophosphate, 2 mM Na₂VO₄, 1 mM DTT, 2.5 mM

MgCl₂, 20 μg/ml aprotinin, 20 μg/ml leupeptin, and 1 mM PMSF. Both ERK1 and ERK2 proteins were immunoprecipitated at 4°C using anti-ERK1 or anti-ERK2 antibody (Santa Cruz Biotechnology), which cross-reacted with both ERK1 and ERK2 proteins, and then with protein G Sepharose (Pierce, Rockford, IL) for 1 h. These complexes were washed three times with PBS containing 1% NP-40 and 2 mM Na₃VO₄, once with Tris buffer (100 mM Tris-Cl, pH 7.5, 500 mM LiCl), and then once with kinase reaction buffer (12.5 mM MOPS, pH 7.5, 12.5 mM β-glycerophosphate, 7.5 mM MgCl₂, 0.5 mM EGTA, 0.5 mM NaF, 0.5 mM Na₃VO₄).

To assay ERK activity, the immunocomplexes were incubated with kinase reaction buffer containing 1 μCi γ-[³²P]ATP, 20 mM unlabeled ATP, 3.3 mM DTT, and 1.5 mg/ml myelin basic protein (MBP, Sigma) as a substrate for ERK at 30°C for 20 min. The samples were then suspended in Laemmli electrophoresis sample buffer, heated at 100°C for 5 min, and analyzed by SDS-PAGE. Using gel slices for each sample, radioactivity was determined with a liquid scintillation counter (LS 6000IC; Beckman Instrs., Fullerton, CA).

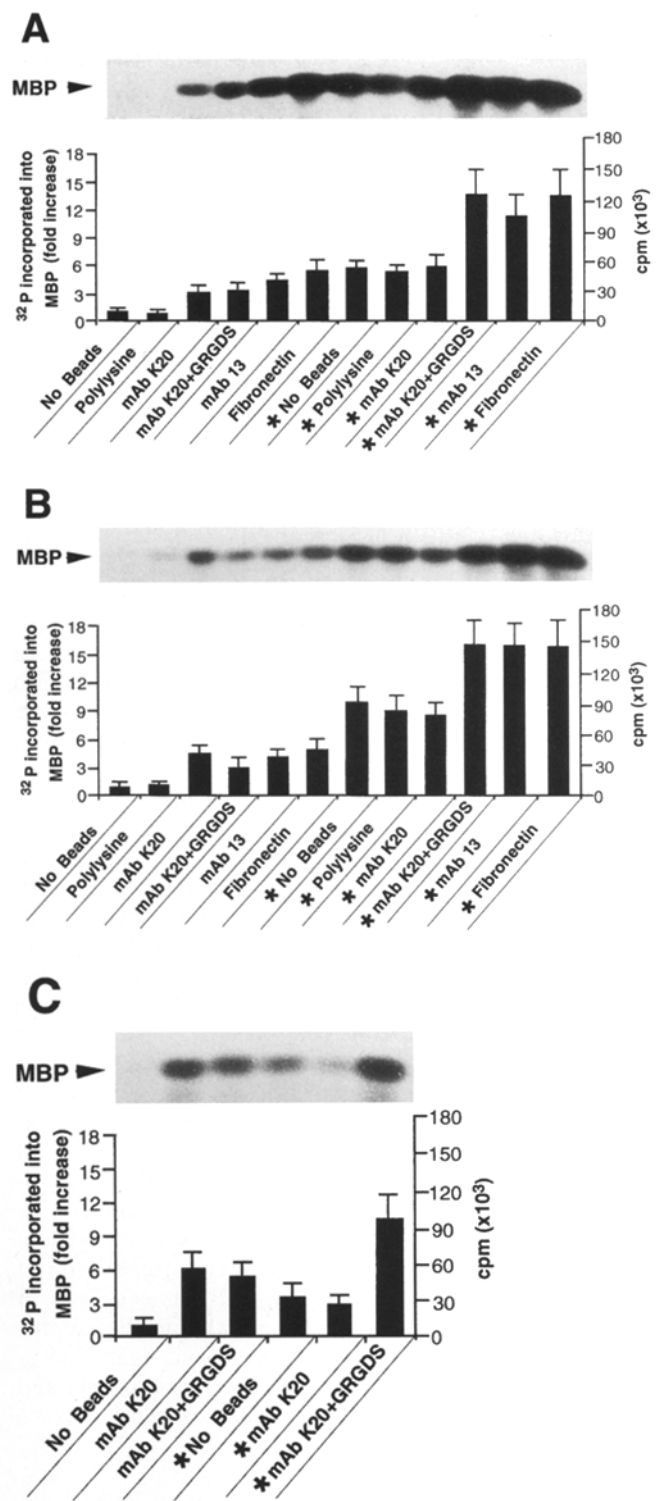
Results

Collaborative Activation of MAP Kinases by Integrins and Growth Factors

MAP kinase activation was explored as a key regulatory pathway potentially involved in integrin-growth factor synergy. Integrins and growth factors are each known to trigger separately the activation of the ERK class of MAP kinase. Such activation of the ERK class of MAP kinase is shown for human fibroblasts in Fig. 1 using EGF, PDGF, or bFGF (Fig. 1, *asterisks*). As previously reported (Miyamoto et al., 1995b), simple clustering of integrins with an aggregating, but not anti-functional antibody on beads (mAb K20), could also induce transient ERK activation, as could fibronectin- and anti-functional antibody (mAb 13)-coated beads (Fig. 1). However, the stimulation of ERK activity by EGF, PDGF-BB, or bFGF was not enhanced for any of these growth factors by costimulation with integrin aggregation mediated by the monoclonal antibody K20 on beads; i.e., the two mechanisms of ERK activation were unexpectedly not additive for EGF (Fig. 1 A), PDGF-BB (Fig. 1 B), or bFGF (Fig. 1 C).

In contrast, if such antibody-aggregated integrins were also incubated with the synthetic peptide Gly-Arg-Gly-Asp-Ser (GRGDS) containing the key RGD sequence to induce ligand occupancy, a large transient activation of ERK activity was then induced after addition of EGF (Fig. 1 A), PDGF-BB (Fig. 1 B), or bFGF (Fig. 1 C). A similar strong activation was observed when growth factors were added under other conditions thought to induce both integrin occupancy and aggregation, i.e., anti-functional antibody- or fibronectin-coated beads (Fig. 1, A and B). This cooperative activation occurring in the presence of integrin aggregation plus occupancy was substantially larger than that obtained without integrin stimulation even with 10 ng/ml of each growth factor, and it was often larger than even the sum of EGF and integrin-mediated activation

Figure 1. Synergistic activation of ERK by integrin aggregation plus ligand occupancy in cooperation with growth factors. Cells were preincubated for 20 min at 37°C with beads coated with the ligands listed in each figure. The cells with attached beads were then incubated with (*asterisks*) or without each growth factor at 10 ng/ml for 5 min at 37°C; the growth factors tested were EGF (A), PDGF-BB (B), and bFGF (C). Activation of ERK was as-



sayed by autoradiography of the myelin basic protein band (MBP, above each graph) and by scintillation counting of incorporated radioactivity. Conditions compared included: absence of beads (*No Beads*), polylysine-coated beads (*Polylysine*), non-inhibitory anti-β₁ mAb K20-coated beads (*mAb K20*), non-inhibitory anti-β₁ mAb K20-coated beads coincubated with GRGDS peptide at 500 μg/ml for 1 h (*mAb K20+GRGDS*), adhesion-blocking anti-β₁ mAb 13-coated beads (*mAb 13*), or human plasma fibronectin-coated beads (*Fibronectin*); asterisk denotes costimulation with growth factor for 5 min after 20 min preincubation of cells with ligand-coated beads.

levels; it was thus strongly additive or weakly synergistic. Similar results were obtained in three experiments.

Requirements for Integrin-mediated Transmembrane Accumulation of Phosphorylated Proteins and EGF-Receptor Substrate

Since ERK and other signaling molecules can be induced to accumulate in transmembrane fashion using ligand-coated beads (Miyamoto et al., 1995; Plopper et al., 1995), we examined for accumulation of total phosphotyrosine-containing proteins and of a growth factor substrate. This localized transmembrane accumulation of tyrosine-phosphorylated proteins required only simple integrin aggregation, as evaluated using a non-inhibitory anti-integrin antibody on beads to induce aggregation and a monoclonal anti-phosphotyrosine antibody for detection (Fig. 2 A). Addition of integrin RGD peptide ligand or the use of fibronectin had relatively modest effects on the extent of accumulation, and the presence of EGF resulted in a particularly strong total accumulation of tyrosine-phosphorylated proteins (Fig. 2 and data not shown). This result appears consistent with the previously reported accumulation of a number of signal transduction molecules at sites of integrin aggregation even in the absence of integrin occupancy, though enhanced in amplitude by occupancy (Miyamoto et al., 1995; Plopper et al., 1995).

A target of EGF receptor-mediated phosphorylation termed eps8 has been implicated in regulation of cell proliferation and malignancy (Fazioli et al., 1993; Matoskova et al., 1995). In marked contrast to the EGF receptor itself, in the absence of EGF treatment this EGF receptor substrate did not accumulate at sites of integrin aggregation above basal levels; no enhanced accumulation was seen even with integrin occupancy, and even under conditions with intact fibronectin on the beads (Fig. 2 B). However, if the EGF receptor was occupied by its growth factor ligand EGF, eps8 accumulated significantly more effectively if conditions of both aggregation and occupancy of integrins were also fulfilled (Fig. 2 B, asterisks). The magnitude of eps8 accumulation was greater if the dose of EGF was increased (Fig. 2 B). The accumulation of eps8 induced by EGF and integrin collaboration was transient, with a marked decline in responsiveness to EGF after 60 min of continuous integrin stimulation (Fig. 2 B, center). Thus, in order to accumulate most effectively, this EGF-receptor substrate requires integrin aggregation and ligand occupancy, as well as growth factor receptor occupancy, and the enhancement of ability to accumulate is transient.

Requirements for Integrin-mediated Growth Factor Receptor Aggregation

We attempted to trace the requirements for MAP kinase activation and eps8 aggregation back to possible physical changes mediated by integrins in the localization of growth factor receptors themselves. Integrin clustering at sites of fibroblast contact with ligand-coated beads was found to be accompanied by local accumulation of each of the three growth factor receptors examined (Figs. 3 and 4). EGF receptors were found to accumulate in a ring around fibronectin-coated beads (Fig. 3 A). Similarly, anti-func-

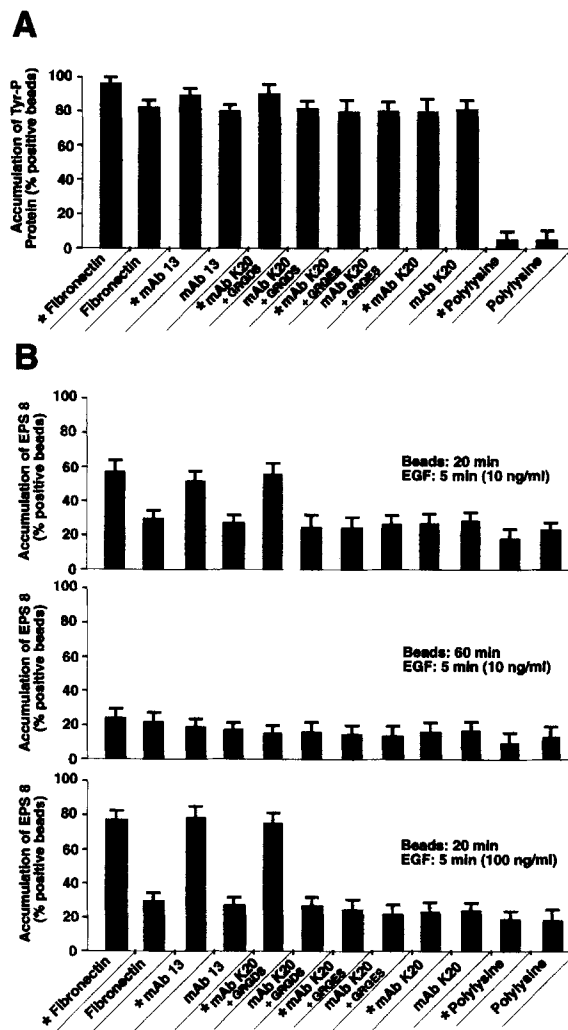


Figure 2. Quantitation of bead-induced accumulation of tyrosine-phosphorylated proteins (A) or epidermal growth factor receptor substrate 8 (eps8; B) with or without EGF stimulation as assessed by immunofluorescence. After 20 min or 60 min preincubation with beads, cells were treated with (asterisks) or without 10 ng/ml or 100 ng/ml human recombinant epidermal growth factor (EGF) for 5 min before fixation for immunofluorescence localization. The ordinate indicates the percentage of beads positive for immunofluorescence accumulation of tyrosine-phosphorylated proteins (Tyr-P) or eps8 using the types of test beads listed along the abscissa; values indicate mean and standard deviation as determined using "blind" counts of transmembrane accumulation of eps8 immunofluorescence adjacent to beads. Polystyrene beads were coated with one of the following ligands: fibronectin, adhesion-blocking antibody mAb 13 to the β_1 subunit, non-inhibitory antibodies K20 to the β_1 subunit, or polylysine. Where indicated, cells were preincubated with GRGDS or GREGS peptide for 1 h at 37°C before the addition of mAb K20-coated beads. Asterisk denotes costimulation with EGF for 5 min after preincubation with ligand-coated beads for 20 or 60 min.

tional antibodies (postulated to provide both aggregation and integrin occupancy) induced a localized accumulation of EGF receptors (Fig. 4). However, simple aggregation of integrins by an aggregating monoclonal antibody on beads did not induce such aggregation of growth factor receptors (Figs. 3 C and 4). On the other hand, if this integrin-medi-

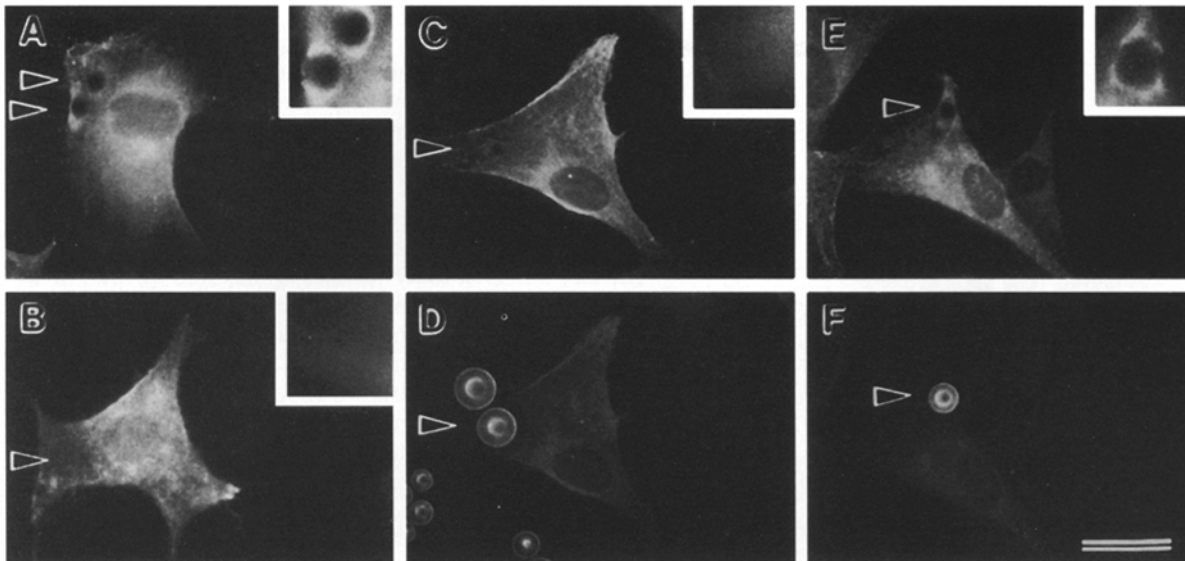


Figure 3. Integrin-induced aggregation of epidermal growth factor receptor (EGF-R) by individual ligand-coated beads. Immunofluorescence localization of EGF-R was determined after incubation with beads coated with fibronectin, polylysine, or mAb K20. Each inset shows a higher magnification view focusing on the equator of the beads marked by the highlighted black arrowheads. Fibronectin-coated beads induced localization of EGF-R when assayed at 20 min (A). Absence of EGF-R localization adjacent to either polylysine-coated beads (B) or mAb K20-coated beads (C) was observed. To visualize the bead, the same cell incubated with mAb K20-coated beads shown in C is shown using combined transmitted light and fluorescence to illuminate the bead (D). After incubation with GRGDS peptide, immunolocalization of EGF-R was found adjacent to mAb K20-coated beads (E); the same bead is also shown illuminated by transmitted light (F). Bar, 20 μ m.

ated aggregation was combined with occupancy with RGD (but not RGE)-containing peptides, or by using fibronectin as the ligand on beads, a similar striking clustering of EGF receptors was observed (Figs. 3 E and 4). Similarly, accumulations of both PDGF α -receptors and PDGF β -receptors, as well as FGF receptors, were induced by integrin interactions with ligands (Fig. 4). In all of these three additional cases, both integrin aggregation and occupancy were necessary preconditions, as determined by analyses using aggregating antibodies and RGD vs RGE peptides (Fig. 4 A: compare +GRGDS with +GRGES), and beads coated with fibronectin or anti-functional vs non-inhibitory antibodies (Fig. 4 A). The integrin-induced accumulation of growth factor receptors was progressively and markedly reduced after prolonged periods of integrin-ligand interaction (Fig. 4 B); the effects on growth factor receptor localization were thus also transient.

Synergistic Growth Factor Receptor Phosphorylation

A recent study described the induction of PDGF β -receptor phosphorylation by integrin clustering in the absence of any growth factor ligand (Sundberg and Rubin, 1996). The effects on MAP kinase activation that we observed might therefore be related to additive receptor phosphorylation. Under our conditions used in this study, however, integrin interaction with fibronectin resulted in little or no detectable increase in tyrosine phosphorylation of either EGF or PDGF receptors (Fig. 5, A and C, lanes 1–3), even though FAK phosphorylation was stimulated by fibronectin. Addition of the corresponding growth factor produced

the expected increase in receptor tyrosine phosphorylation in the presence or absence of bound polylysine-coated beads (Fig. 5, A and C, lanes 4 and 5). Interestingly, however, the combination of fibronectin-coated beads and growth factors resulted in a synergistic enhancement of the tyrosine phosphorylation of the cognate receptor. This effect was observed for both EGF receptors and PDGF receptors (lane 6 in Fig. 5, A and C). Controls using the same blots reprobbed with antibody against either the EGF receptor or the PDGF β -receptor confirmed equal loading of lanes (Fig. 5, B and D).

This synergistic enhancement of growth factor receptor tyrosine phosphorylation was not observed with a combination of EGF with simple integrin aggregation mediated by non-inhibitory antibody K20 plus EGF, even though integrin aggregation alone was able to stimulate FAK phosphorylation as previously reported (e.g., see Yamada and Miyamoto, 1995; Fig. 5 E, lanes 3 and 8). Instead, synergistic stimulation of EGF receptor tyrosine phosphorylation required a combination of three conditions: EGF treatment, integrin ligand occupancy, and antibody-induced aggregation (compares lane 9 with lanes 3–5, and 8 in Fig. 5 E). Substitution of a peptide with a conservative amino acid substitution (GRGES) for the GRGDS peptide resulted in a loss of the synergistic receptor phosphorylation, even though FAK tyrosine phosphorylation remained (Fig. 5 E, lane 10). Very similar results were obtained when the same conditions were compared for phosphorylation of PDGF receptors mediated by PDGF-BB, i.e., both integrin aggregation and occupancy were necessary for synergistic tyrosine phosphorylation of PDGF receptors (Fig. 5 F).

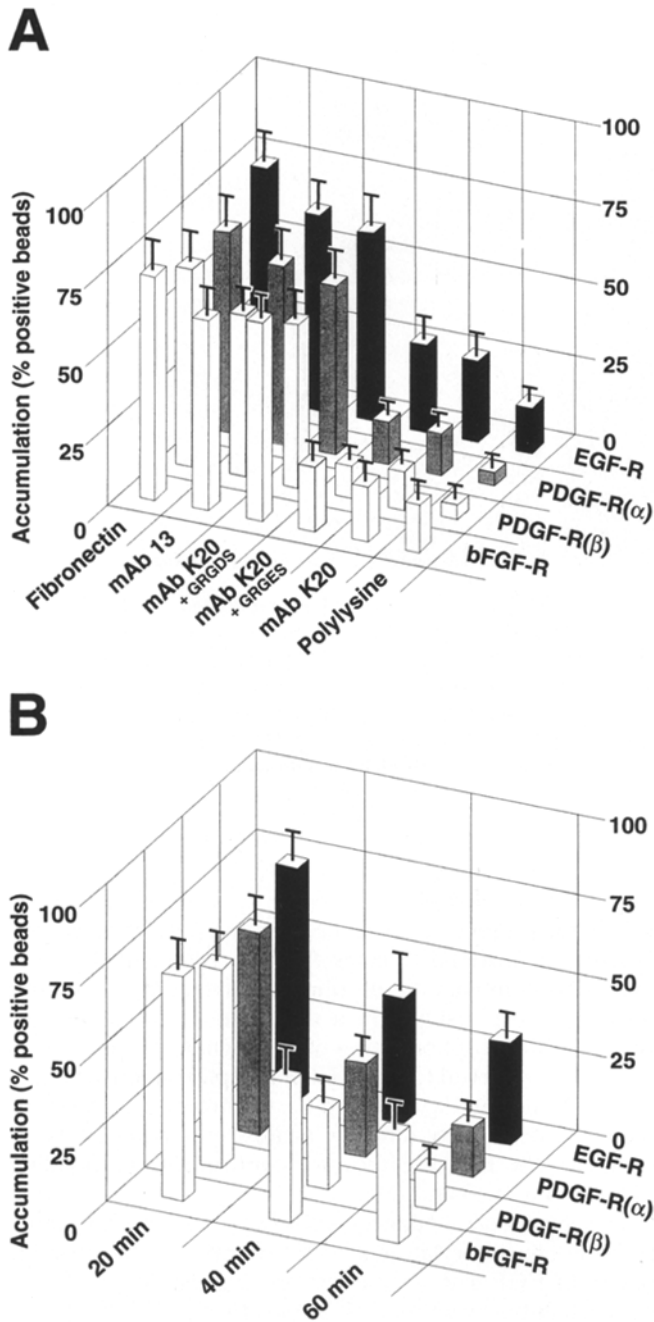


Figure 4. Quantitation of bead-induced accumulation of EGF-receptor, PDGF α -receptor, PDGF β -receptor, or bFGF-receptor as assessed by immunofluorescence microscopy. The ordinate indicates the percentage of beads positive for immunofluorescence for each antigen listed on the right; values indicate mean and standard deviation. In *A*, the different types of beads or beads plus soluble peptide are listed along the bottom of the figure. Polystyrene beads were coated with one of the following ligands: fibronectin, adhesion-blocking antibody mAb 13 to the integrin β_1 subunit, non-inhibitory antibody K20 to the β_1 subunit, or polylysine. To examine the role of ligand occupancy, cells were preincubated with GRGDS or control GRGES peptide for 1 h at 37°C before the addition of mAb K20-coated beads. *B* shows the transient nature of the clustering of growth factor receptors. Fibronectin-coated beads were incubated with the cells for 20, 40, or 60 min before examination for clustering of the four different receptor antigens listed on the right.

Discussion

Integrin-mediated signaling is a complex process. This study has examined the requirements for integrin induction of growth factor receptor and substrate accumulation in signaling complexes, as well as for potential synergism between the actions of integrins and growth factors on receptor phosphorylation and MAP kinase activation. Major new conclusions from our study include the following: (1) The ERK class of MAP kinases is activated by integrin aggregation alone or by various growth factors, but the two are not additive in the absence of integrin occupancy. However, in the presence of both integrin aggregation and occupancy, there is a striking increase in ERK activity with the growth factor ligands EGF, PDGF, or bFGF. (2) Although the local accumulation of bulk tyrosine-phosphorylated proteins requires only integrin aggregation and not occupancy, maximal accumulation of EGF-receptor substrate 8 (eps8) requires both integrin aggregation and occupancy, as well as the ligand EGF itself. (3) Each of the different growth factor receptors, for EGF, PDGF (α - and β -receptors), and bFGF, are induced to accumulate at sites of integrin-ligand aggregation. This process does not require external growth factor ligand, but it does require both integrin aggregation and occupancy. (4) Synergy with integrins also occurs at the level of growth factor receptor phosphorylation: receptor tyrosine phosphorylation is only minimally enhanced by integrin action, is stimulated by growth factors, and is enhanced markedly by the combination of integrin and growth factor.

A schematic summary of our findings is presented in Fig. 6, indicating three levels of response to stimulation of integrin or growth factor receptors. This collaborative response involving growth factor receptor phosphorylation, substrate accumulation, and MAP kinase activation provides new mechanisms relevant to synergism at a biological level between specific growth factors and integrin receptors.

The autophosphorylation of growth factor receptors of the tyrosine kinase family is thought to be induced by ligand-mediated dimerization (Fantl et al., 1993; Lemmon and Schlessinger, 1994; Heldin, 1995). Although clustering of such receptors by antibodies is often sufficient to trigger autophosphorylation and the downstream effects induced by ligand binding, simple dimer formation may not be sufficient, and the process may require effects of ligand binding on receptor subunit orientation or conformation (Gadella and Jovin, 1995). Downstream stimulation of ERK in the MAP kinase pathway is well-characterized for this class of receptor kinases, and the activation of ERK results in a variety of effects on gene regulation, growth, and differentiation (e.g., see reviews by Blumer and Johnson, 1994; Johnson and Vaillancourt, 1994; Treisman, 1996).

The previously best-characterized mechanism for growth factor and integrin synergy involves the separate actions of cell surface receptors that bind fibronectin (presumably integrins) and the growth factor PDGF on enzyme and substrate in inositol lipid metabolism to generate enzyme-substrate synergism (McNamee et al., 1993). Particularly relevant findings, however, are the recently reported coaccumulation of a variety of signaling molecules such as a growth factor receptor for FGF (Plopper et al., 1995) and

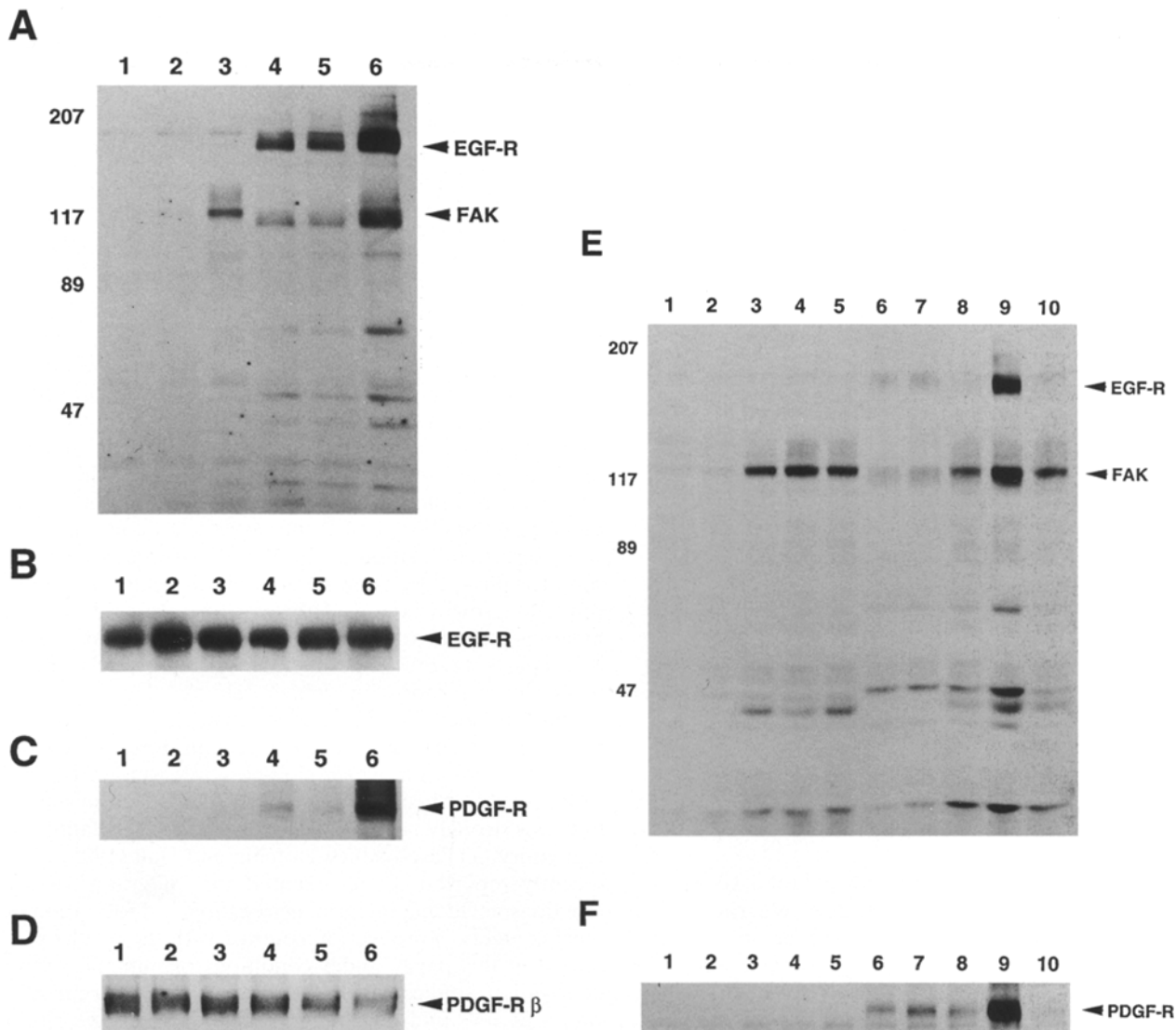


Figure 5. Synergistic effects on growth factor receptor tyrosine phosphorylation induced by integrin aggregation plus ligand occupancy in cooperativity with growth factors. Cells were incubated with ligand-coated beads for 20 min at 37°C. The cell-beads complexes were treated with each growth factor for 5 min at 37°C, and protein tyrosine phosphorylation was determined by Western immunoblotting with monoclonal anti-phosphotyrosine (A, C, E, and F). Cells were incubated without growth factors (lanes 1–3 of A–D) or stimulated with 10 ng/ml EGF (lanes 4–6 of A and B) or PDGF-BB (lanes 4–6 of C and D) after preincubation with the following: control with no beads (lanes 1 and 4); with polylysine-coated beads (lanes 2 and 5), or with fibronectin-coated beads (lanes 3 and 6). The electrophoretic mobility and total amount of EGF receptor (B) or PDGF β -receptor (D) protein was analyzed in each lane by stripping and reanalyzing the same immunoblot with the appropriate antibody; similar amounts were found for each condition. In E and F, roles of integrin aggregation and occupancy in the synergism for receptor tyrosine phosphorylation were examined. Cells were incubated without growth factors (lanes 1–5) or stimulated with 10 ng/ml EGF (lanes 6–10 of E) or PDGF-BB (lanes 6–10 of F) after preincubation with the following: control with no beads (lanes 1 and 6); with polylysine-coated beads (lanes 2 and 7), with aggregating non-inhibitory mAb K20 beads (lanes 3 and 8); with mAb K20 plus GRGDS (lanes 4 and 9); or with mAb K20 plus the control peptide GRGES (lanes 5 and 10).

MAP kinases (Miyamoto et al., 1995b) in bead-induced transmembrane cytoskeletal and signaling complexes. Ingber and others have suggested that such accumulations could be centers of signaling, since receptor enzyme and substrate are brought into close proximity in these large accumulations of cytoskeletal and signaling molecules at integrin-induced adhesion sites that may form discrete functional complexes (Turner and Burridge, 1991; Damsky and Werb, 1992; Fazioli et al., 1993; Carraway and Carraway, 1995; Jockusch et al., 1995; Geiger et al., 1995;

Plopper et al., 1995; Yamada and Miyamoto, 1995; Sundberg and Rubin, 1996). The present study establishes that integrin stimulation can induce a similar accumulation of FGF, EGF, and PDGF α - and PDGF β -receptors in fibroblasts, and that it requires not only integrin aggregation, but also ligand occupancy. This latter finding suggests that occupancy may be needed for conformational unmasking or exposure of the β_1 integrin cytoplasmic tail for additional cytoplasmic interactions, as suggested by previous studies on altered function after occupancy that appear to

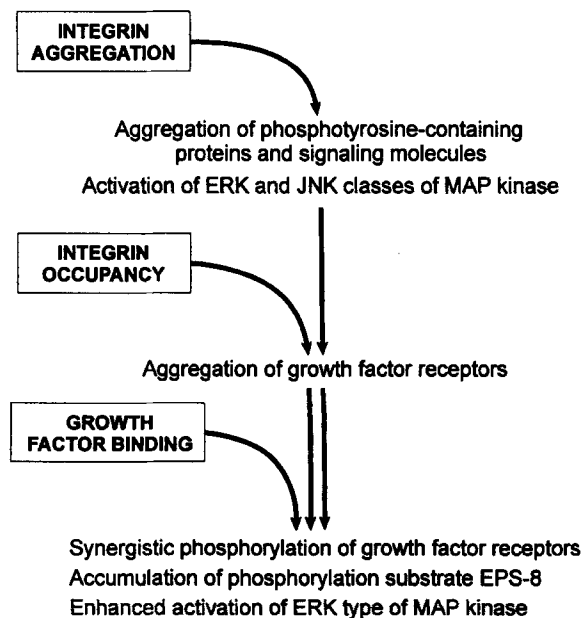


Figure 6. Collaborative or synergistic interactions of integrin and growth factor receptors. Schematic listing of the sequential cooperative effects of simple integrin aggregation with integrin occupancy, and then growth factor receptor occupancy on localization and function of several types of signal transduction molecule, based on data presented in this study.

be independent of aggregation (LaFlamme et al., 1992; Schwartz et al., 1995).

It was of interest that accumulation of total tyrosine-phosphorylated proteins did not require integrin occupancy and was not accompanied by growth factor receptor clustering. This finding suggests that simple aggregation of proteins with phosphotyrosine residues is not sufficient to induce accumulation of tyrosine kinase growth factor receptors, which instead required concurrent integrin occupancy. The mechanism of accumulation of growth factor receptors remains to be determined, but it is noteworthy that these same conditions were needed for integrin-mediated transmembrane accumulation of cytoskeletal molecules such as talin, α -actinin, and actin (Miyamoto et al., 1995b; Yamada and Miyamoto, 1995b). Evidence for interactions of EGF receptors with cytoskeletal molecules has been reported in several previous studies, and the subpopulation associated with the cytoskeleton appears to be enriched in high-affinity EGF receptors (Roy et al., 1989; van Bergen en Henegouwen et al., 1992; Gronowski and Bertics, 1993). These findings suggest that the accumulation of growth factor receptors in the absence of growth factor ligands or receptor autophosphorylation might be mediated by cytoskeletal interactions.

The addition of growth factor ligand to a system containing pre-aggregated EGF receptors leads to substantially increased accumulation of the EGF receptor substrate eps8, a protein implicated in downstream growth regulation and malignant transformation (Fazioli et al., 1993; Matoskova et al., 1995). However, eps8 molecules did not accumulate above basal levels along with the EGF receptor if EGF ligand was not present. That is, even though in-

tegrin occupancy and aggregation is sufficient to induce accumulation of EGF receptors, enhanced accumulation of the EGF receptor substrate eps8 requires in addition the presence of EGF. A simple explanation might be that local accumulation of this substrate regulatory molecule occurs in proximity to the EGF receptor only after EGF receptor autophosphorylation.

The findings of strongly augmented activation of the ERK class of MAP kinase by integrin aggregation and occupancy when they are combined with EGF, PDGF, or bFGF are striking, and they demonstrate that a well-known signaling pathway can show collaborative activation by growth factors and integrins. This type of response may contribute to downstream biological effects of such combinations; this possibility should be examined in any biological system where collaboration or synergism between growth factor and integrin is found. The simplest mechanism of such MAP kinase activation would be via enhanced receptor tyrosine phosphorylation. In agreement with Sundberg and Rubin (1996), we could not observe direct stimulation of the PDGF α -receptor or EGF receptor phosphorylation by an integrin ligand in the absence of growth factors. However, we also did not see major growth factor-independent stimulation of PDGF β -receptors in our experimental system, which differs markedly in protocol by deliberately avoiding long-term serum starvation. We chose to maintain cells in serum until the final steps in order to try to remain closest to normal culture conditions in which biological integrin-growth factor synergy had been described. In addition, serum starvation can strongly suppress function of the Rho family of regulatory GTPases, which Hotchin and Hall (1995) have recently reported to be essential for integrin-mediated functions, including integrin aggregation and formation of focal contacts. Moreover, in contrast with the results presented in this paper under conditions of minimal serum deprivation, studies performed after extensive serum starvation of cells find that integrins cannot synergize with a growth factor for MAP kinase activation, or in one report cannot even activate the ERK1/2 MAP kinase pathway (Zhu and Assoian, 1995; Hotchin and Hall, 1995). Although extensively serum-deprived fibroblasts appear to require both integrin-mediated anchorage and growth factor stimulation for cell cycle progression and synergy in inositol lipid breakdown, other processes such as induction of *c-myc* mRNA and phosphorylation of phospholipase C_γ do not show synergistic effects (McNamee et al., 1993; Bohmer et al., 1996).

The strong stimulation we observed of tyrosine phosphorylation of growth factor receptors after stimulation by a growth factor in cooperativity with integrin aggregation and occupancy was demonstrated for two different types of growth factor receptor. The mechanism of the synergistic enhancement of growth factor tyrosine phosphorylation described here is probably steric: the integrin-mediated aggregation of growth factor receptors before growth factor occupancy would transiently concentrate these tyrosine kinase receptors locally, which would be expected to enhance their capacity for transphosphorylation. It is interesting that MAP kinase activation by integrin aggregation alone without occupancy was not additive with growth factor activation. This result suggests that they share a

rate-limited biochemical pathway. In contrast, when integrin aggregation and occupancy leads to transient growth factor receptor clustering, there is striking synergy of receptor tyrosine phosphorylation, and a downstream activation of the ERK class of serine/threonine kinase.

The magnitude of the response to integrin-growth factor collaboration in tyrosine kinase receptor phosphorylation appeared stronger than the downstream activation of MAP kinase. Similar nonlinearity between receptor and downstream signaling has been described in other systems (e.g., see Vaillancourt et al., 1995), and can be ascribed to the existence of multiple phosphorylation sites on the receptors associated with differing downstream signaling systems.

The transience that we observe in growth factor receptor clustering, EGF receptor substrate clustering, and MAP kinase response may be important for maintaining responsiveness and flexibility of this integrin-mediated signaling system. For example, it might permit rapid, transient signaling responses to local changes in extracellular matrix composition or to new growth factors as cells migrate to different locations, or when levels of these molecules change during embryonic development or during tissue repair and remodeling.

In summary, we have identified a system in which growth factors and integrins can collaborate directly at the levels of tyrosine kinase receptor phosphorylation and downstream MAP kinase activation. This synergy with each of three well-known growth factors in turn depends on both integrin aggregation and receptor occupancy. This direct functional cooperation between major regulatory systems provides a mechanism by which growth factors and integrins can synergize to mediate complex biological processes.

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