Molecular Biology of the Cell
Vol. 6, 1349–1365, October 1995

Convergence of Integrin and Growth Factor Receptor Signaling Pathways within the Focal Adhesion Complex

George E. Plopper,* Helen P. McNamee, Laura E. Dike, Krzysztof Bojanowski, and Donald E. Ingber†

Departments of Surgery and Pathology, Children's Hospital and Harvard Medical School, Boston, Massachusetts 02115

Submitted February 21, 1995; Accepted July 24, 1995
Monitoring Editor: Richard Hynes

Extracellular matrix controls capillary endothelial cell sensitivity to soluble mitogens by binding integrin receptors and thereby activating a chemical signaling response that rapidly integrates with growth factor-induced signaling mechanisms. Here we report that in addition to integrins, growth factor receptors and multiple molecules that transduce signals conveyed by both types of receptors are immobilized on the cytoskeleton (CSK) and spatially integrated within the focal adhesion complex (FAC) at the site of integrin binding. FACs were rapidly induced in round cells and physically isolated from the remainder of the CSK after detergent-extraction using magnetic microbeads coated with fibronectin or a synthetic RGD-containing peptide. Immunofluorescence microscopy revealed that multiple signaling molecules (e.g., $pp60^{c\text{-}src}$, $pp125^{FAK}$, phosphatidylinositol-3-kinase, phospholipase C-γ, and Na^+/H^+ antiporter) involved in both integrin and growth factor receptor signaling pathways became associated with the CSK framework of the FAC within 15 min after binding to beads coated with integrin ligands. Recruitment of tyrosine kinases to the FAC was also accompanied by a local increase in tyrosine phosphorylation, as indicated by enhanced phosphotyrosine staining at the site of integrin binding. In contrast, neither recruitment of signaling molecules nor increased phosphotyrosine staining was observed when cells bound to beads coated with a control ligand (acetylated low density lipoprotein) that ligates transmembrane scavenger receptors, but does not induce FAC formation. Western blot analysis confirmed that FACs isolated using RGD-beads were enriched for $pp60^{c\text{-}src}$, $pp125^{FAK}$, phospholipase C-γ, and the Na^+/H^+ antiporter when compared with intact CSK or basal cell surface preparations that retained lipid bilayer. Isolated FACs were also greatly enriched for the high affinity fibroblast growth factor receptor *flg*. Most importantly, isolated FACs continued to exhibit multiple chemical signaling activities in vitro, including protein tyrosine kinase activities ($pp60^{c\text{-}src}$ and $pp125^{FAK}$) as well as the ability to undergo multiple sequential steps in the inositol lipid synthesis cascade. These data suggest that many of the chemical signaling events that are induced by integrins and growth factor receptors in capillary cells may effectively function in a "solid-state" on insoluble CSK scaffolds within the FAC and that the FAC may represent a major site for signal integration between these two regulatory pathways. Future investigations into the biochemical and biophysical basis of signal transduction may be facilitated by this method, which results in isolation of FACs that retain the CSK framework as well as multiple associated chemical signaling activities.

* Present address: Department of Cell Biology/SBR-12, The Scripps Research Institute, La Jolla, CA 92037.

† Corresponding author: Enders 1007, Children's Hospital, 300 Longwood Ave., Boston, MA 02115.

INTRODUCTION

Angiogenesis, the growth of capillary blood vessels, is critical for normal embryogenesis and plays a central role in many pathological conditions, including cancer (Ingber, 1992). Angiogenesis is also representative of many morphogenetic systems in that it is controlled through interplay between three different types of external stimuli: soluble growth factors, insoluble extracellular matrix (ECM) molecules, and mechanical stresses (Ingber and Folkman, 1989a; Ingber, 1991). In general, soluble mitogens activate intracellular chemical signaling pathways that are required for growth whereas ECM and mechanical influences control cell sensitivity to these soluble cues and govern whether individual capillary cells will grow, differentiate, or undergo apoptosis and involute in the local tissue microenvironment (Ingber *et al.*, 1986; Ingber and Folkman, 1989b; Ingber, 1992; Brooks *et al.*, 1994). A similar form of signal integration has been observed in many other developing tissues (Damsky and Werb, 1992; Schwartz and Ingber, 1994). However, the mechanism by which cells process these diverse inputs and produce a single, concerted response remains unclear.

Analysis of the molecular basis of growth regulation has revealed that binding interactions between ECM and cell surface integrin receptors play a critical role in coordinating this response in many cell types, including capillary endothelial cells (Ingber and Folkman, 1989a; Schwartz *et al.*, 1991; Schwartz and Ingber, 1994; Damsky and Werb, 1992; Brooks *et al.*, 1994). Integrins are heterodimeric transmembrane receptors comprised of α and β subunits. At least 20 different integrin types have been identified that mediate cell attachment to a wide variety of ECM molecules and cell surface proteins (Hynes, 1992). In response to binding to immobilized ligands, many integrins cluster and become immobilized within specialized "focal adhesion" complexes (FACs) due to binding interactions with actin-associated cytoskeletal proteins (e.g., talin, vinculin, α-actinin, and paxillin) (Burridge *et al.*, 1988). This molecular bridge between ECM, integrins, and actin microfilaments stabilizes cell adhesion and provides a continuous path for mechanical signal transfer across the cell surface (Ingber, 1991; Wang *et al.*, 1993).

Cell attachment to ECM is also known to activate at least five different classes of chemical signaling pathways that share common downstream targets with growth factor signaling mechanisms. Signaling molecules that have been shown to be specifically activated by adhesion to ECM and integrin binding include the following: tyrosine kinases (e.g., $pp60^{c\text{-}src}$ and $pp125^{FAK}$; Guan *et al.*, 1991; Kornberg *et al.*, 1992; Lipfert *et al.*, 1992; Schlaepfer *et al.*, 1994) and serine/threonine kinases (e.g., protein kinase C, p34/cdc2, MAP kinase, and cyclin A kinase; Chun and Jacobson, 1992; Symington, 1992; Davis *et al.*, 1993; Vuori and Ruoslahti, 1993; Chen *et al.*, 1994); proteins that mediate inositol lipid turnover (Banga *et al.*, 1986; Cybulsky *et al.*, 1993; McNamee *et al.*, 1993); G proteins (Torti *et al.*, 1991; Kapron-Bras *et al.*, 1993); and ion transporters, including calcium channels (Pelletier *et al.*, 1992; Schwartz, 1993) and the Na^+/H^+ antiporter (Ingber *et al.*, 1990; Schwartz *et al.*, 1991). Activation of these signaling molecules by cell binding to either ECM or growth factors may also feed back to alter integrin function. Activators of protein kinase C modulate FAC assembly in multiple cell types (Rahilly and Fleming, 1992; Woods and Couchman, 1992) and growth factors, such as platelet-derived growth factor (PDGF) and lysophosphatidic acid, promote FAC formation and cytoskeleton (CSK) reorganization by activating small molecular weight, rho-like G proteins (Ridley and Hall, 1992). ECM-dependent activation of phosphatidylinositol-4-phosphate (PIP) kinase activity (McNamee *et al.*, 1993) may also play a role in actin polymerization by altering phosphatidylinositol-4,5-bis-phosphate (PIP_2) levels and thereby modulating actin interactions with profilin and/or gelsolin (Janmey and Stossel, 1987; Goldschmidt-Clermont *et al.*, 1990).

Despite the fact that they share common downstream targets, integrin signaling pathways and growth factor-associated cascades are distinct and complementary. For example, ECM and the soluble angiogenic mitogen, basic fibroblast growth factor (FGF), act independently to activate the Na^+/H^+ antiporter when they bind capillary endothelial cells (Ingber *et al.*, 1990). Although ECM binds to integrins and FGF binds to its own high affinity cell surface receptor (*flg*) (Zhan *et al.*, 1993), they produce additive effects on Na^+/H^+ exchange within minutes after binding and both stimuli are required for optimal cell proliferation (Ingber *et al.*, 1990). The control of inositol lipid turnover is another example of distinct, but integrated, signaling mechanisms. In fibroblasts, PDGF stimulates the release of inositol phosphates from inositol lipids by activating phospholipase C-γ (PLC) whereas ECM regulates the availability of the inositol lipid substrate PIP_2 by controlling its synthesis (McNamee *et al.*, 1993). Again, signal integration occurs within minutes after receptor binding and under all conditions, growth factors and ECM must work hand-in-hand to produce the full cellular response. It is therefore critical to understand how cells simultaneously integrate signals elicited by these different stimuli.

It is well known that many soluble growth factors alter cell growth and function by binding to transmembrane receptors on the cell surface and thereby unmasking tyrosine kinase activity within the cytoplasmic portion of those receptors. Resulting changes in tyrosine phosphorylation and molecular configura-

tion expose binding sites that mediate direct associations with many other types of intracellular signaling molecules (e.g., PLC, $pp60^{c\text{-}src}$, and GAP; Koch *et al.*, 1991). In this manner, the growth factor receptor is thought to represent a critical site for signal transduction. In general, integrins contain short cytoplasmic domains and have no endogenous kinase activity (Hynes, 1992). Thus, little is known about how integrin binding activates all of the different classes of signaling molecules that were described above. Even less is known about how binding of integrins at the cell base can modulate transduction events (e.g., inositol lipid breakdown) that are controlled by growth factor receptors at different sites on the cell surface.

A clue to this mechanism came from the observation that integration of the signals from growth factors and ECM occurs rapidly after integrin binding. For example, synthesis of the inositol lipid PIP_2 can be detected within 2 min (McNamee *et al.*, 1993), activation of the Na^+/H^+ antiporter can be detected within 10 min (Ingber *et al.*, 1990), and induction of protein tyrosine phosphorylation can be detected within 20 min (Guan and Shalloway, 1991; Kornberg *et al.*, 1992) following cell binding to ECM in many cells, including capillary cells. This rapid response suggested to us that signal integration could occur locally within the FAC at the site of integrin binding. It is well known that many viral tyrosine kinases (e.g., $pp60^{v\text{-}src}$) localize to FACs in transformed cells (Cooper and Hunter, 1981; Kellie *et al.*, 1986a,b; Tapley *et al.*, 1989; Turner *et al.*, 1989; Guan and Shalloway, 1992; Lipfert *et al.*, 1992) and that phosphotyrosine (PTyr)-containing proteins localize to FACs within normal cells (Marchisio *et al.*, 1984; Maher *et al.*, 1985). Furthermore, one integrin-associated focal adhesion kinase, $pp125^{FAK}$, exhibits both growth factor and ECM-dependent phosphorylation on tyrosine (Guan *et al.*, 1991; Kornberg *et al.*, 1992; Zachary and Rozengurt, 1992) and physically associates with $pp60^{c\text{-}src}$ via its SH2 domain when activated (Schaller *et al.*, 1992; Xing *et al.*, 1994; Cobb *et al.*, 1994). $pp125^{FAK}$ is a substrate for $pp60^{v\text{-}src}$, along with many other FAC proteins (Rohrschneider *et al.*, 1982; Kellie *et al.*, 1986b; Tapley *et al.*, 1989; Guan and Shalloway, 1992) and a recent report suggests that integrin-dependent phosphorylation of $pp125^{FAK}$ may link integrin engagement to the Ras/MAP kinase signal transduction pathway via creation of an SH2-binding site for GRB2 (Schlaepfer *et al.*, 1994). The distribution of $pp125^{FAK}$ within FACs as well as its ability to be activated by growth factors appears, however, to be more dependent on its associations with the actin CSK (Lipfert *et al.*, 1992; Rankin and Rozengurt, 1994). Another FAC component, tensin (Davis *et al.*, 1991), contains an SH2 domain as well as an actin-binding motif and hence, provides a potential mechanism to immobilize PTyr-containing proteins (e.g., PLC, ras GAP, and src family members) to the actin CSK in these regions. It is important to emphasize that protein tyrosine kinases are not the only signaling molecules that localize at the site of integrin binding. Protein kinase C, a signaling molecule that is activated by many growth factors and is downstream in the inositol signaling cascade, also localizes to the FAC in certain cells (Jaken *et al.*, 1989).

Taken together, these observations suggested to us that integrins may integrate a variety of different signaling pathways that are activated by both ECM and growth factors by promoting formation of the specialized CSK scaffold that forms the backbone of the FAC. This scaffold (i.e., rather than the lipid bilayer) would, in turn, serve to orient the chemical signaling molecules that mediate these transduction events in close proximity and hence, provide efficient cross-talk and signal integration. Although immunolocalization studies imply that chemical signaling occurs at the site of integrin binding, direct evidence is lacking because there is currently no way to carry out biochemical analysis of signaling specifically within the FAC. To address this limitation, we adapted a recently described method that utilizes magnetic microbeads coated with integrin ligands to induce rapid FAC formation and to isolate these insoluble complexes from the remainder of the cell and CSK (Plopper and Ingber, 1993). Using this approach, we now show that FACs that form in response to capillary cell binding to these microbeads contain molecules involved in many different types of signaling pathways as well as a growth factor receptor. Most importantly, these purified FACs continue to exhibit diverse chemical signaling activities in vitro, even after removal of membranes and soluble cytosolic proteins. These results support our hypothesis that integrins act as signal integrators by spatially orienting active chemical signalling molecules and bringing them into close proximity within the FAC. The findings also imply that the current concept of chemical signaling occurring on the "membrane" (i.e., within the lipid bilayer) may need to be revised to include "solid-state" channeling along insoluble CSK scaffolds.

MATERIALS AND METHODS

Experimental System

Bovine capillary endothelial cells isolated from adrenal cortex were routinely cultured in DMEM supplemented with 10% calf serum, glutamine (0.3 mg/ml), penicillin (100 U/ml), streptomycin (100 μg/ml), 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), pH 7.4, and 10 μg/ml endothelial mitogen (Biomedical Technologies, Stoughton, MA). Two days before experiments, cells were cultured in DMEM containing 1% serum and no endothelial mitogen to induce quiescence and remove soluble growth factors. Quiescent cell monolayers were dissociated by brief exposure to trypsin-EDTA, collected by centrifugation, washed in 1% bovine serum albumin (BSA)/DMEM, and plated for experiments in defined medium (Ingber *et al.*, 1991) without FGF. In studies in which metabolic radiolabeling was required, confluent monolayers

of cells were cultured for 24 h in labeling medium (10% calf serum dialyzed against phosphate-buffered saline (PBS)/20 mM HEPES, pH 7.4/10 μg/ml endothelial mitogen/90% methionine-free DMEM) containing [^{35}S]methionine (10 uCi/ml; 1140 Ci/mmol, NEN, Boston, MA). To achieve quiescence, this medium was replaced with an identical medium containing 1% dialyzed calf serum for 2 days after the initial labeling period. Tosyl-activated magnetic microbeads (4.5 um diameter; Dynal, Oslo, Norway) were coated (all at 50 μg/ml in 0.1 M carbonate buffer, pH 9.4) with FN, RGD peptide (Peptite 2000, Telios, San Diego, CA) or acetylated low density lipoprotein (AcLDL; Biomedical Technologies) for 24 h at 4°C, as previously described (Schwartz *et al.*, 1991; Plopper and Ingber, 1993). Beads were stored in 1% BSA/DMEM/20 mM HEPES before use.

Immunofluorescence Microscopy

For immunofluorescence studies, cells were cultured (7,500 cells/cm^2) for 18 h in defined medium without FGF on 8-well Labtek glass culture slides (VWR) coated with a low density of FN (25 ng/well) that promotes cell attachment but not cell spreading, as previously described (Ingber, 1990). Coated microbeads were added to each well (20 beads/cell) of the Labtek slides and allowed to incubate for 15 or 30 min at 37°C. To identify cytoskeletal-associated FAC proteins, cells were incubated for 1 min in ice cold CSK stabilizing buffer [CSK-TX; 50 mM NaCl, 150 mM sucrose, 3 mM $MgCl_2$, 20 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM phenylmethylsulfonyl fluoride, 10 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid), pH 6.8] that maintains the integrity of the CSK (Burr *et al.*, 1980), followed by 1 min in the same buffer supplemented with 0.5% Triton X-100 (CSK+TX) to remove membranes and soluble cytoplasmic (non-CSK) components. Detergent-extracted cells were fixed in 4% paraformaldehyde/PBS, washed with PBS, and incubated with primary antibodies diluted in 0.2% Triton X-100/0.1% BSA in PBS. Primary antibodies were visualized using affinity-purified rhodaminated goat anti-rabbit, sheep anti-mouse, or rabbit anti-sheep antibodies (Organon Teknika-Cappel, Durham, NC). Fluorescence images were photographed on a Zeiss Axiomat microscope (Thornwood, NY) using Kodak Tri-X-Pan film.

Isolation of FACs

FACs were isolated from suspended cells as previously described (Plopper and Ingber, 1993). In brief, cells dispersed with trypsin-EDTA were washed in 1% BSA/DMEM and suspended (10^6 cells/ml) in defined medium without FGF except for some *flg* studies in which FGF (5 ng/ml) was included. Magnetic RGD-beads (2×10^7/ml) were added to the cell suspension and rotated for 30 min at 37°C. RGD-coated beads were utilized because they exhibited less nonspecific clumping in suspension than FN-beads and thus, allowed greater binding efficiency. However, similar results have been obtained with FN-beads. Subsequent procedures were carried out at 4°C using a side pull magnetic separation unit (Advanced Magnetics, Cambridge, MA) to collect microbeads and bound cells. Bead-bound cells were washed in CSK-TX buffer, transferred into CSK+TX buffer, sonicated for 10 s (tune and power settings = 4; W 10 sonicator, Heat Systems Ultrasonics), and homogenized in a 2 ml dounce homogenizer (Wheaton) with a clearance of 50–100 micrometers (20 strokes). Microbeads were magnetically pelleted and washed five times with 10 ml CSK+TX buffer. RIPA buffer (1% Triton X-100, 1% deoxycholate, 0.1% SDS, 150 mM NaCl, 50 mM Tris, pH 7.2, 0.1 mM AEBSF [4-(2-Aminoethyl)-benzenesulfonylfluoride, HCl]) was used to remove protein from beads for biochemical analysis.

Intact CSK and basal cell surfaces were isolated from adherent capillary cell monolayers, as previously described (Plopper and Ingber, 1993). In brief, the intact CSK was collected by extracting monolayers with the CSK+TX buffer, as described above, followed by scraping and pelleting of the insoluble material. The basal cell surface fraction was prepared using the wet-cleaving (WC) method of Brands and Feltkamp (1988). Nitrocellulose paper (0.45 μm pore; Schleicher & Schuell, Keene, NH) was wetted in water, placed on top of the endothelial cell monolayer for 15 s, and then quickly removed with forceps, thereby "wet cleaving" the cells and removing the apical cell surfaces. The remaining adherent basal cell surfaces were scraped into ice cold PBS containing 1 mM aprotinin using a rubber policeman and pelleted by centrifugation (5 min, $1500 \times g$) at 4°C. This procedure does not include detergent, and thus, lipid bilayers in the basal plasma membrane should remain intact.

Western Blot Analysis

Proteins dissolved in RIPA buffer were separated by SDS-PAGE (7.5% acrylamide) and transferred to nitrocellulose (0.45 μm pore; Schleicher & Schuell). In all blotting studies, equal protein (50 μg) from different experimental samples was placed within each lane, as determined using a BCA protein microassay (Pierce, Rockford, IL). Nonspecific binding sites on the nitrocellulose were blocked with 0.5% casein in Tris-buffered saline before incubation with primary antibodies followed by affinity-purified horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit secondary antibodies (Organon Teknika-Cappel). Labeled proteins were visualized using an ECL chemiluminescence kit (Amersham, Arlington Heights, IL). Quantitation was carried out using a Datacopy GS Plus scanning densitometer (Xerox) as described (Masters *et al.*, 1992).

Antibodies

Rabbit serum against ankyrin and affinity-purified rabbit antibodies against nonmuscle myosin were purchased from East Acres Biologicals (Southbridge, MA) and Biomedical Technologies, respectively. Rabbit polyclonal antibodies were obtained against the src gene product (using Rous sarcoma virus as an antigen) from East Acres Biologicals and Calbiochem (La Jolla, CA). A third sheep polyclonal antibody directed against a c-src peptide from the human pp60$^{c\text{-}src}$ amino acid sequence (Arg-Glu-Val-Leu-Asp-Gln-Val-Glu-Arg-Gly-Tyr-Arg-Met-Pro-Cys; Tanaka *et al.*, 1987) was obtained from Cambridge Research Biochemicals (now sold by Genosys); all antibodies produced identical results (the data presented were obtained using the Calbiochem antibody). Rabbit polyclonal antibodies against PLC and PI-3-kinase were obtained from Upstate Biotechnology (Lake Placid, NY). Mouse monoclonal and rabbit polyclonal antibodies against pp125^{FAK} were a generous gift of Dr. Tom Parsons (University of Virginia, Charlottesville, VA). Mouse monoclonal antibodies against PTyr were kindly provided by Dr. Morris White (Joslin Diabetes Center, Boston, MA) or purchased from ICN, and rabbit polyclonal antibodies against P-Tyr were generously provided by Dr. Tom Roberts (Dana-Farber Cancer Institute, Boston, MA) or purchased from Upstate Biotechnology; all of these antibodies gave similar results. Mouse monoclonal antibodies against *flg* were kindly provided by Dr. Tom Maciag (Red Cross, Bethesda, MD). The binding specificity of these anti-*flg* antibodies has been previously characterized in detail (Zhan *et al.*, 1993,1994); we also carried out positive controls and confirmed that these antibodies recognized recombinant *flg* protein using our Western blot procedure. Affinity-purified rabbit antibodies generated against a cytoplasmic peptide (EPLAYDPCDC, residues 704–712) of the published sequence of the human Na^+/H^+ antiporter were generously provided by Dr. Martin A. Schwartz (Scripps Institute, La Jolla, CA). This antibody recognized proteins with apparent molecular weights of 110 kDa and 66 kDa from 10T1/2 fibroblasts and bovine endothelial cells, respectively, when analyzed by immunoblotting or immunoprecipitation, as previously reported for other anti-Na^+/H^+ antiporter antibodies (Ross *et al.*, 1990; Sardet *et al.*, 1990).

In Vitro Protein Kinase Assays

For analysis of total protein kinase activity, isolated bead-associated FACs were washed in kinase buffer (10 mM Tris, pH 7.4, 5 mM

$MnCl_2$) and incubated at 37°C in the same buffer containing 100 uCi/ml [γ-^{32}P]ATP (3000 Ci/mmol, NEN). The reaction was stopped by washing the beads in kinase buffer lacking radiolabel and boiling for 100°C for 5 min in fourfold concentrated (4×) SDS gel sample buffer. The in vitro activities of specific kinases were measured after immunoprecipitation of the relevant proteins. Proteins contained in extracts from whole cells, intact CSK, and isolated FACs were solubilized in RIPA buffer with protease inhibitors, clarified by centrifugation, and the supernatant was precleared by addition of protein A-sepharose beads, followed by centrifugation. Five microliters of antibody were added per sample, and samples were allowed to mix at 4°C for 18 h. Protein A-sepharose beads (25 μl/sample) were added for the final 2 h. Samples were pelleted by centrifugation and washed in RIPA buffer and kinase buffer before beginning the in vitro kinase assay. pp125^{FAK} activity, was measured by quantitating autophosphorylation of pp125^{FAK}, as described by Hildebrand *et al.* (1993). After completion of the kinase reaction, samples were boiled in SDS sample buffer, separated by SDS-PAGE using 7.5% acrylamide gels, dried, and exposed to x-ray film. pp60$^{c\text{-}src}$ kinase activity was measured in vitro using enolase as a substrate, as described by Clarke and Brugge (1993). In brief, after the immunoprecipitates of c-src were washed in kinase buffer, the kinase reaction was initiated with addition of 5 uCi [γ-^{32}P]ATP (3000 Ci/mmol, NEN), 1 mM unlabeled ATP, and 5 μg enolase. After incubation at 37°C for 15 min, the reaction was stopped by addition of 1/4 volume of 4× SDS gel sample buffer and boiling at 100°C for 5 min. Reaction products were separated by SDS-PAGE, and the specific activity of pp60$^{c\text{-}src}$ was determined by comparing the incorporation of ^{32}P into enolase in different samples that were normalized on an equal protein basis.

PIP Kinase Assay

Lipid kinase assay methods were adapted from Ling *et al.* (1989). Samples were diluted before the assay to reduce the concentration of detergents; the final assay buffer contained 30 mM Tris, pH 7.4, 0.1% TX-100, 0.1% deoxycholate, 0.01% SDS, 15 mM NaCl, 1 mM dithiothreitol, 0.1 mM AEBSF, 1 mM EDTA, 0.1 M EGTA, 80 uM PI4P, 80 uM phosphoserine, 100 uM ATP, 5 mM $MgCl_2$, and 2 uCi/ml [γ-^{32}P]ATP (3000 Ci/mmol). Samples were incubated at room temperature for 5 min after addition of ATP, and then reactions were stopped by addition of ice cold methanol:1 M HCL (1:1). Lipids were extracted by addition of one-half volume of chloroform. The organic phase was then extracted again with an equal volume of methanol:1 mM EDTA (1:0.9) and dried under nitrogen stream. The isolated lipids were spotted onto oxalate-pretreated silica gel thin layer chromatography plates (LK6DF; Whatman, Maidstone, UK) and separated using chloroform:methanol:4 M NH_4OH (9:7:2). Labeled lipids were visualized by autoradiography and identified by comparison to ^{3}H-labeled inositol lipid standards (NEN) run in parallel, as previously described (McNamee *et al.*, 1993). PI-3-kinase activity was measured by immunoprecipitating the enzyme using a specific antibody (#06–195; Upstate Biotechnology) and carrying out an in vitro kinase assay using PI as a substrate according to the supplier's recommended protocol.

RESULTS

Signaling Molecules Are Rapidly Recruited to the FAC Following Integrin Binding

Immunofluorescence microscopy was used in conjunction with microbeads (4.5-um diameter) coated with two different integrin ligands, fibronectin (FN), or a synthetic RGD-containing peptide (Peptite 2000), to determine whether signaling molecules are recruited to the FAC during the first 15 to 30 min after ECM binding, under conditions when growth-associated signaling pathways become activated. We have previously shown that, in contrast to certain other cell types (e.g., hepatocytes; Hansen *et al.*, 1994), capillary endothelial cells spread and grow equally well on dishes coated with FN or RGD-peptide (Ingber *et al.*, 1995). Furthermore, binding of microbeads coated with both integrin ligands induces FAC formation, transfers mechanical signals to the CSK, and activates chemical signaling pathways in capillary cells over the same time course and to the same degree as cell binding to ECM-coated planar dishes (Ingber *et al.*, 1990; Schwartz *et al.* 1991; Plopper and Ingber, 1993; Wang *et al.*, 1993; and our unpublished data). To specifically focus on associations with the CSK framework of the FAC, cells bound to beads were extracted with detergent (0.5% Triton X-100) in a CSK-stabilizing buffer to remove membranes and soluble cytosolic components before fixation. Past studies have shown that cellular proteins do not nonspecifically associate with the cytoskeleton when extracted with Triton under these conditions (Burr *et al.*, 1980; Fey *et al.*, 1984; Ball, 1986).

Immunofluorescence staining confirmed that FACs formed at the site of integrin binding within 15 min after cells were bound to RGD-beads, as indicated by enhanced staining for vinculin and talin along the bead-cell interface (Figure 1). We have previously obtained similar results using FN-beads and have shown that these bead-induced FACs also contain integrin β1, α-actinin, and paxillin (Plopper and Ingber, 1993). This FAC staining appeared to be specific because CSK proteins that do not normally localize to focal adhesions, such as nonmuscle myosin and ankyrin, did not become preferentially concentrated along the cell-bead interface (Figure 1). Specific induction of FAC formation in response to cell binding to microbeads coated with integrin ligands also has been demonstrated by other laboratories in different cell types (Lewis and Schwartz, 1995; Miyamoto *et al.*, 1995).

Importantly, immunofluorescence staining of CSK preparations extracted and fixed in the same manner confirmed that the tyrosine kinases pp60$^{c\text{-}src}$ and pp125^{FAK} were also recruited to the CSK at the site of FN-bead binding (Figure 2). The FAC staining pattern appeared as a crescent along the bead-cell interface by 15 min after bead addition and then progressed to form a halo completely surrounding these FN-coated beads as they began to be internalized over the next 30 min to 3 h. Recruitment of these tyrosine kinases also was accompanied by a concomitant increase in PTyr staining on the CSK at the surface of these beads (Figure 2), a finding consistent with previous studies that demonstrate that cell binding to FN induces tyrosine phosphorylation of a number of proteins, including pp125^{FAK}, over a similar time course (Guan *et al.*, 1991; Kornberg *et al.*, 1992). In addition,

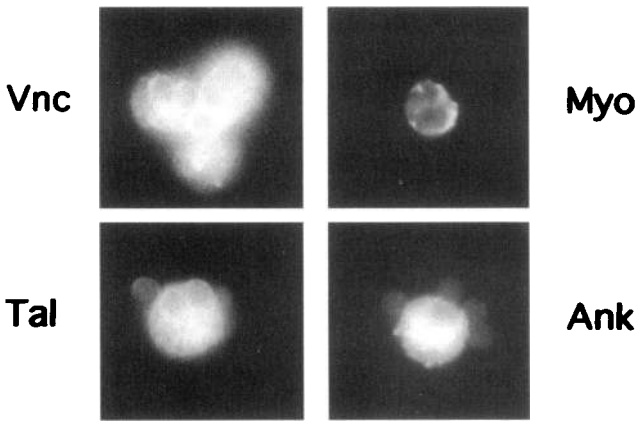

Figure 1. Cell binding to beads coated with integrin ligands induces FAC formation. Immunofluorescence micrographs showing that the focal adhesion proteins, vinculin (Vnc) and talin (tal), are recruited to the cell-bead interface within 30 min after RGD-bead binding whereas two other cytoskeletal components that are not normally found in focal adhesions, nonmuscle myosin (myo) and ankyrin (Ank), are not. Beads are 4.5 um in diameter.

we observed similar bead staining for two enzymes that are involved in inositol lipid signaling by growth factors, PLC and phosphatidylinositol-3-kinase (PI-3-kinase), as well as an ion channel that is a downstream target for both integrin- and growth factor receptor-dependent signaling pathways, the Na^+/H^+ antiporter (Figure 2). Similar staining patterns were observed using RGD-beads. In contrast, none of the signaling molecules were recruited to the cell-bead interface when cells bound beads coated with AcLDL (Figure 2). AcLDL-beads bind avidly to specific transmembrane receptors ("scavenger" receptors; Kume *et al.*, 1991) on the capillary cell surface and are internalized over a similar time course. However, AcLDL-beads do not induce FAC formation (as detected by immunofluorescence microscopy; Plopper and Ingber, 1993) or activate integrin-dependent signaling pathways (Schwartz *et al.*, 1991). Mechanical measurements show that AcLDL-beads only physically associate with elements of the elastic, submembranous CSK (cortical ankyrin-spectrin-actin lattice) whereas beads coated with integrin ligands link directly to the FAC and hence, become structurally coupled to the internal CSK framework of the cell (i.e., the interconnected central network of microfilaments, intermediate filaments, and microtubules; Wang *et al.*, 1993; Wang and Ingber, 1994).

Many Signaling Molecules Are Enriched within Isolated FACs

To confirm that the signaling molecules that appeared to be CSK-associated were enriched at the site of integrin binding, we analyzed FACs that were magnetically isolated from the remainder of the insoluble CSK. Cells that were allowed to bind to RGD-beads for 30 min in suspension were extracted with buffer containing Triton X-100 to remove membranes and soluble cytosolic components, sonicated to disrupt CSK continuity, and Dounce homogenized to remove residual nuclei. The bead-associated FACS were isolated from the remainder of the insoluble debris by taking advantage of the magnetic nature of the beads. This procedure results in a FAC preparation that is greatly enriched for known FAC proteins, including integrin $\beta 1$, talin, vinculin, α-actinin, and paxillin (Plopper and Ingber, 1993). For example, in this study, vinculin was enriched in the FAC fraction by 10-fold compared with the whole CSK and enriched 4.5-fold relative to a "wet-cleaved" preparation of basal cell surfaces that retains both lipid bilayer and submembranous CSK (Brands and Feltkamp, 1988), when equal protein samples were analyzed by immunoblotting (Figure 3A) and densitometric quantitation (Figure 3B). In contrast, two primary internal CSK components, nonmuscle myosin (Figure 3A) and actin (Plopper and Ingber, 1993) were relatively depleted in the FAC fraction relative to the basal cell surface and CSK preparations (by more than 80 and 90%, respectively; Figure 3B). Thus, these peripheral adhesion complexes appeared to be effectively separated from the internal CSK.

The specificity of the FAC isolation procedure was also demonstrated by the finding that magnetic isolation of protein complexes that formed in response to cell binding to AcLDL-beads under identical conditions resulted in protein recovery of less than 0.5% of that obtained with RGD-beads, even though both beads bind avidly to specific transmembrane receptors on the CE cell surface (Plopper and Ingber, 1993). Comparison of protein profiles within the complexes that were magnetically isolated using RGD-beads versus AcLDL-beads also confirmed that they exhibited different protein compositions. For example, when bead complexes were isolated from ^{35}S-methionine-labeled cells and compared on an equal cpm basis, only two major protein species were visualized in the AcLDL-bead complex compared with over 30 in the FAC (Figure 4), a result that is consistent with our immunfluorescence results (Figures 1 and 2). Nonspecific protein binding to beads also was neglible: less than 0.002% of total cellular protein became associated with the RGD-beads when they were added to ^{35}S-methionine-labeled cells after detergent addition and then carried through the entire FAC isolation procedure (i.e., compared with more than 5% when cells were allowed to bind beads first and were then triton extracted). Thus, FAC formation and associated recruitment of signaling molecules did not appear to

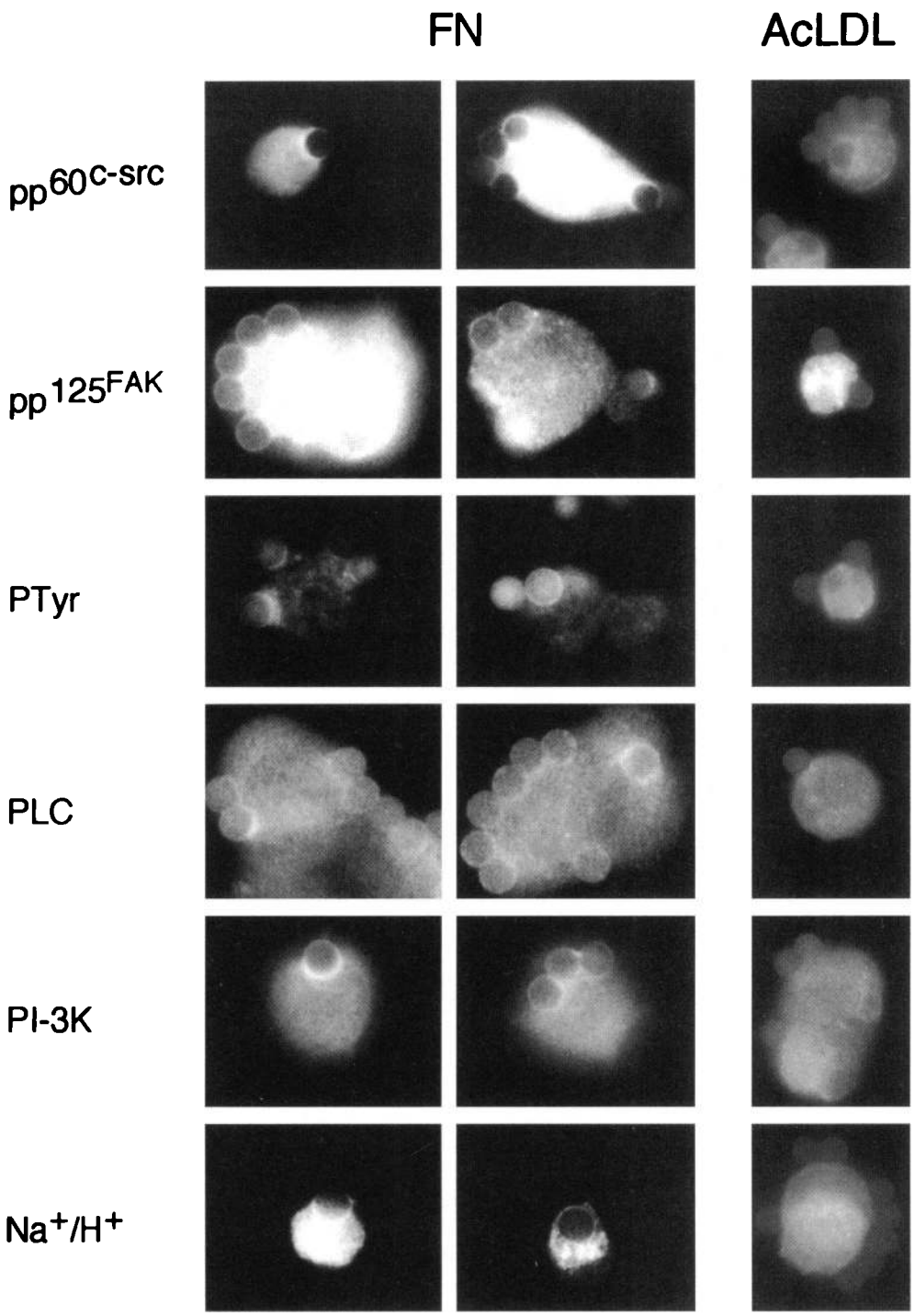

Figure 2. Recruitment of signaling molecules to the FAC in response to cell binding to FN-beads for 15 (left) and 30 min (middle) or to AcLDL-beads for 30 min (right). Only FN-beads induced recruitment of chemical signaling molecules to the bead cell interface (beads are 4.5 um in diameter). PTyr, phosphotyrosine; PLC, phospholipase C-γ; PI-3K, PI-3kinase; Na^+/H^+, Na^+/H^+ antiporter.

be due to nonspecific binding to bead surfaces or to a general effect of substrate adhesion, rather they resulted specifically from cell binding to surfaces coated with integrin ligands.

Western blot analysis of protein fractions prepared during progressive steps in the FAC isolation procedure confirmed that the FAC is the site in which many PTyr-containing proteins localize within the cell (Figure 5). Specifically, analysis of proteins extracted from whole cells revealed that integrin clustering induced by cell binding to FN-coated beads (Schwartz *et al.*, 1991; Plopper and Ingber, 1993) caused an increase in

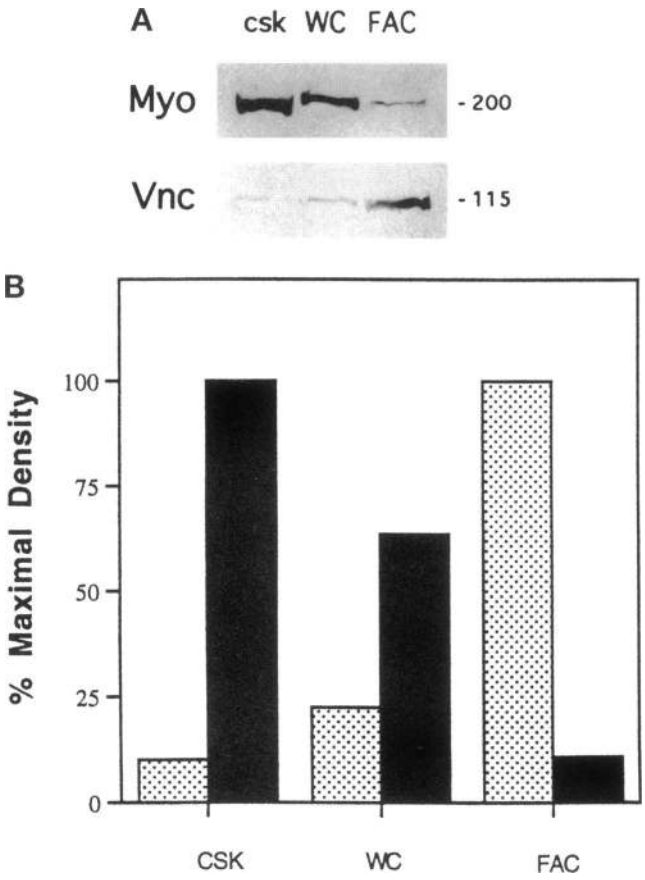

Figure 3. Isolated FACs are enriched for vinculin and depleted for myosin relative to intact CSK and basal cell surface fractions. (A) Western blots using antibodies against myosin (Myo) and vinculin (Vnc). Equal protein samples (50 μg) from the intact CSK (CSK), wet-cleaved basal cell surface (WC), or isolated FAC preparations (FAC) were loaded in each lane. Molecular weight standards are indicated at right. (B) Densitometric quantitation of the blots shown is in panel A. Black bars, myosin; stippled bars, vinculin.

tyrosine phosphorylation, as previously reported in other cell types (Guan *et al.*, 1991; Kornberg *et al.*, 1992), whereas binding to soluble FN and hence, integrin occupancy alone, did not. Furthermore, the vast majority of PTyr-containing proteins in bead-bound cells were CSK-associated and a large subset of these proteins were retained and/or enriched within isolated FACs. Two of the major PTyr-containing protein species exhibited apparent molecular weights of 60 and 120 kDa.

Western blot analysis of equal protein samples confirmed that $pp60^{c\text{-}src}$, PLC, and the Na^+/H^+ antiporter were significantly enriched within the FAC compared with either the intact CSK or basal cell surface preparations (Figure 6A). Densitometric quantitation of these blots revealed that $pp60^{c\text{-}src}$ was enriched approximately threefold in the isolated FAC relative to intact CSK and was nearly undetectable in the basal

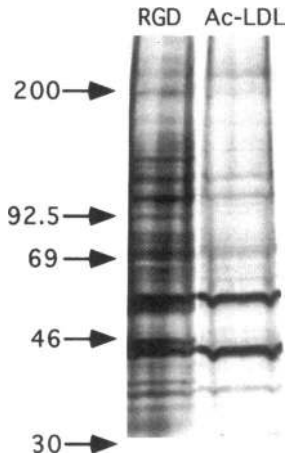

Figure 4. Autoradiograph of an SDS polyacrylamide gel (7.5%) comparing ^{35}S-labeled proteins that were isolated from prelabeled cells using beads coated with RGD peptide versus AcLDL. FAC preparations isolated using RGD-beads contained different and much more varied proteins than complexes isolated using AcLDL-beads.

cell surface fraction whereas PLC (145 kDa) was detected only in the isolated FAC (Figure 6B). In addi-

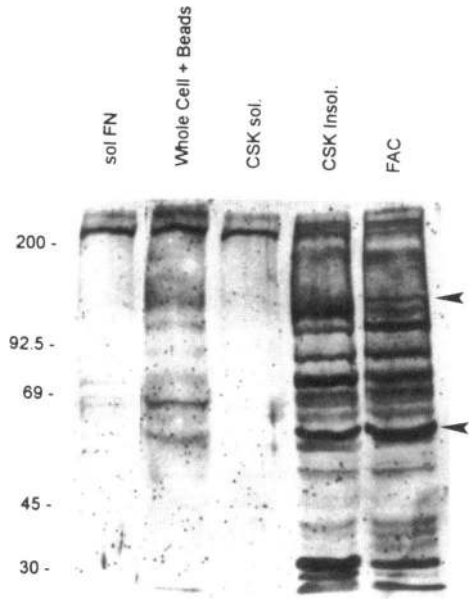

Figure 5. Western blot probed with anti-PTyr antibodies comparing protein collected from suspended cells cultured in the presence of soluble FN with that isolated from cells bound to RGD-beads at different stages in the FAC isolation procedure. Sol FN, total cellular protein isolated from cells incubated with soluble FN (50 μg/ml) for 30 min in suspension; Whole Cell + Beads, total cellular protein isolated from cells incubated with RGD-beads for 30 min in suspension; CSK sol., soluble proteins removed when bead-bound cells were extracted with detergent-containing buffer; CSK insol., CSK proteins removed from the insoluble bead-cell complexes as a result of sonication and dounce homogenization; FAC, remaining insoluble RGD bead-associated protein complex. Upper and lower arrowheads at right indicate protein bands that migrate with apparent molecular weights of 120 and 60 KDa, respectively. Equal protein (50 μg) was loaded in each gel lane; molecular weight standards are indicated at left.

tion, levels of the Na^+/H^+ antiporter were found to be three to five times higher in the isolated FAC relative to the basal cell surface and CSK fractions, respectively. The extraction buffers we used for FAC isolation were adapted from a CSK isolation procedure that was previously used to demonstrate that active $pp60^{v\text{-}src}$ associates with the CSK (Burr *et al.*, 1980). The presence of calcium and magnesium in this buffer is required to preserve the structural integrity of the CSK as well as the FAC (Ball *et al.*, 1986), however, it leaves open the potential for degradation of FAC-associated molecules by cation-dependent proteases. For example, $pp60^{c\text{-}src}$ is a substrate for the calpain II (Oda *et al.*, 1993), a calcium-dependent protease that has been reported to localize to the FAC (Beckerle *et al.*, 1987). In fact, we observed a minor band with a mobility of 47 kDa in our $pp60^{c\text{-}src}$ Western blots (Figure 5A) that corresponds to the size of the cleaved form of this kinase, as previously reported (Oda *et al.*, 1993). Interestingly, a lower (52 kDa) band also was labeled by antibodies against PLC (Figure 6B) in our blots. Others have noted that anti-PLC antibodies can cross-react with a protein of similar mobility that appears to be the SH2/SH3 adaptor protein Nck (Meisenhelder and Hunter, 1992). However, the identity of the protein detected in our blots remains to be determined.

The finding that FACs were enriched for PLC, a signaling molecule that appears to be more sensitive to control by growth factors than ECM (McNamee *et al.*, 1993), suggested that this specialized CSK scaffolding also may serve to orient, and hence coordinate, growth factor receptor-induced signaling events. If this is true, then we might expect that the growth factor receptor itself would be in close proximity to this signaling complex. The high affinity growth factor receptor *flg* mediates mitogenic stimulation by FGF (Zhan *et al.*, 1993). Bovine capillary cells only express a few thousand high affinity FGF receptors on their cell surface (our unpublished observation) and thus, it is very difficult to visualize *flg* in whole cell or intact CSK preparations by Western blot analysis (Figure 7). In contrast, all three different forms of *flg* that migrate with characteristic apparent molecular weights of 110,

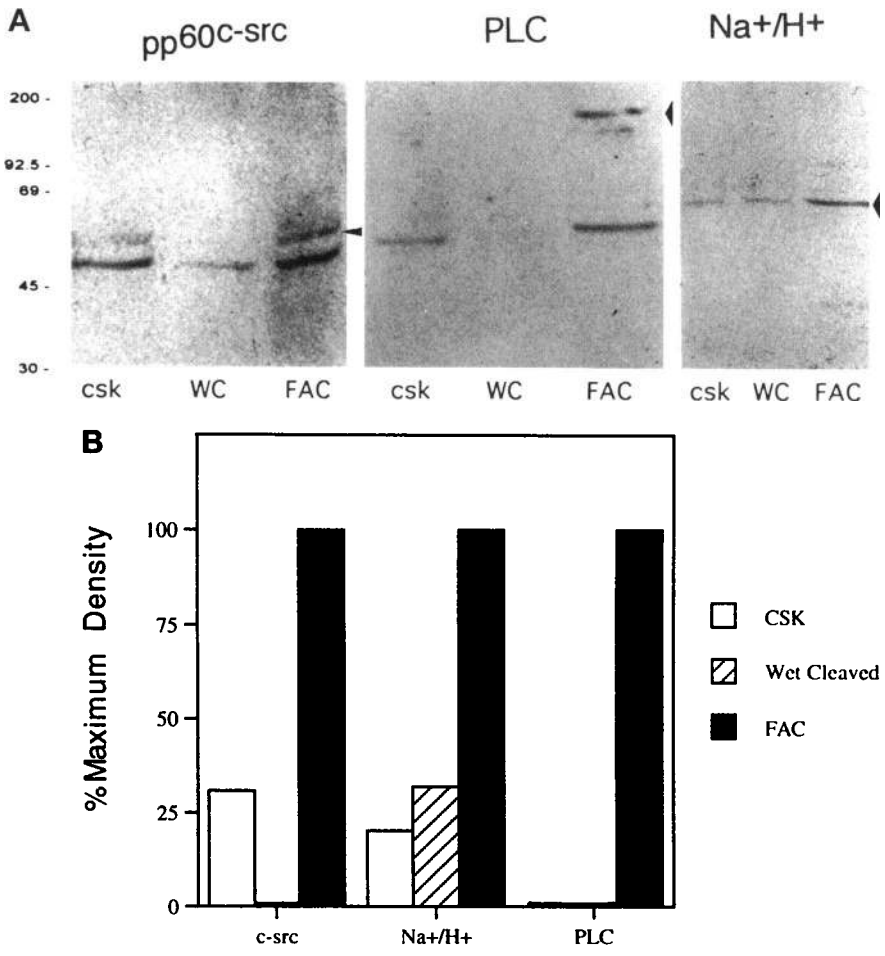

Figure 6. Isolated FACs are enriched in chemical signaling molecules. (A) Western blots of $pp60^{c\text{-}src}$, PLC-γ (PLC), and the Na^+/H^+ antiporter (Na^+/H^+). Equal amounts of protein from intact CSK (csk), wet-cleaved basal cell surface preparation (WC), or isolated FACs (FAC) were run in the left, middle, and right lanes of each gel, respectively, and blotted with the antibodies directed against the indicated signaling molecules. All of the blots are aligned; molecular weight standards are indicated at left. Arrowheads indicate the bands used for densitometric quantitation shown in panel B. The lower molecular weight bands in the $pp60^{c\text{-}src}$ and PLC blots may respectively represent a proteolytic fragment of $pp60^{c\text{-}src}$ (Oda *et al.*, 1993) and the SH2/SH3 adaptor protein Nck (Meisenhelder and Hunter, 1992). (B) Densitometry of blots shown in panel A. Only staining for the higher molecular weight bands were quantitated in the $pp60^{c\text{-}src}$ and PLC blots. Open bar, CSK; striped bar, WC; closed bar, FAC.

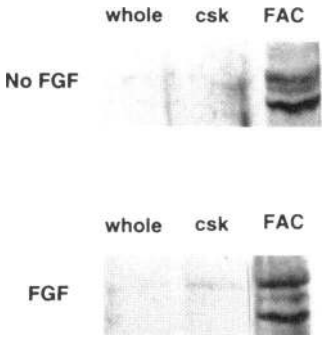

Figure 7. The high affinity FGF receptor *flg* is enriched within isolated FACs compared with whole cells (whole) or intact CSK (csk). Western blot analysis revealed the presence of the 110, 125, and 135 kDa forms of *flg* (Zhan *et al.*, 1993) within the FAC, regardless of whether cells were cultured in the presence (FGF) or absence (No FGF) of soluble FGF. In contrast, *flg* was barely detectable in the whole cell and CSK fractions when analyzed on an equal protein basis.

125, and 135 kDa (Zhan *et al.*, 1993,1994) were easily visualized in Western blots of isolated FAC preparations when compared with whole cell and intact CSK preparations on an equal protein basis (Figure 7). Interestingly, FAC formation induced by integrin binding appeared to be sufficient to recruit and concentrate *flg* within this signaling complex even in the absence of FGF in the medium and thus, without significant growth factor binding (Figure 7).

Isolated FACs Retain Multiple Signaling Activities

Our method for isolating intact FACs offers the potential to retain physiologically relevant spatial relationships between different signaling elements that are normally lost by conventional analytical approaches that are highly disruptive (e.g., immunoprecipitation of solubilized protein extracts). In fact, when intact bead-associated FACs were incubated with [γ-^{32}P]-ATP in vitro, they were found to retain active protein kinases, as detected by the phosphorylation of a wide range of different proteins, whereas protein complexes obtained using AcLDL-beads were completely inactive (Figure 8). Immunoprecipitation of proteins that became labeled in the in vitro kinase assay using anti-PTyr antibodies demonstrated that only a subset of these proteins contained PTyr and that the two most prominent PTyr-proteins exhibited approximate apparent molecular weights of 60 and 120 kDa (Figure 8). One explanation of this result is that the immunoprecipitation procedure using anti-PTyr antibodies may be extremely inefficient. Alternatively, some active kinases that are retained within FACs after magnetic isolation may actually be *non*-tyrosine (e.g., serine/threonine) kinases.

Given that we found pp60$^{c\text{-}src}$ to be enriched within isolated FACs by Western blot analysis (Figure 6A), it was possible that the 60-kDa PTyr-containing protein observed in Figure 8 could represent active pp60$^{c\text{-}src}$ that became autophosphorylated when placed in the in vitro kinase assay. To directly test whether pp60$^{c\text{-}src}$

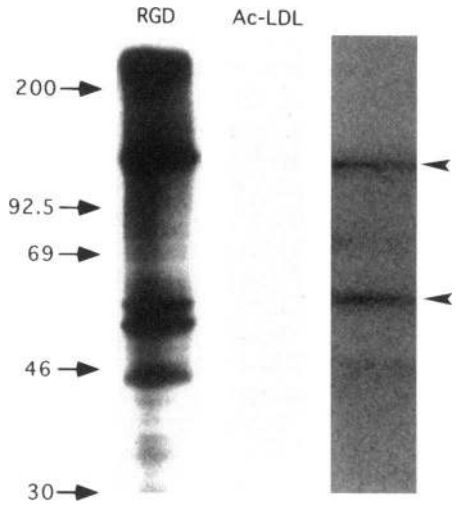

Figure 8. Isolated FACs retain multiple active protein kinases and substrates. Protein complexes isolated using RGD-beads (RGD) retained active protein kinases (left lane) whereas those obtained with AcLDL-beads (Ac-LDL) did not (middle lane). Bead-associated complexes were isolated from cells bound to RGD-beads or AcLDL-beads, exposed to [γ-^{32}P]ATP, and boiled in sample buffer, and proteins were separated by SDS-PAGE (equal cpm loaded per lane). All ^{32}P counts associated with Ac-LDL-bead complexes migrated in the dye front. Right lane, PTyr-containing proteins immunoprecipitated from bead-associated complexes isolated from RGD-beads following in vitro labeling with [γ^{32}P]ATP, as shown in the left lane. Molecular weights of protein standards are shown at the left; upper and lower arrowheads at the right indicate immunoprecipitated ^{32}P-labeled bands migrating with mobilities of approximately 120 and 60 kDa, respectively.

that was present and enriched in the FAC exhibited tyrosine kinase activity, we used an in vitro immunocomplex kinase assay for pp60$^{c\text{-}src}$ that was developed by Clark and Brugge (1993). pp60$^{c\text{-}src}$ was immunoprecipitated from proteins extracted from whole cells (incubated in the presence of either soluble FN or FN-coated beads) or from fractions collected at progressive stages in the FAC isolation procedure. Its kinase activity was analyzed by measuring phosphorylation of enolase, an exogenous src substrate, in the presence of [γ-^{32}P]ATP. When compared on an equal protein basis, phosphorylated enolase (indicating the presence of pp60$^{c\text{-}src}$ activity) could only be detected in reactions that included proteins immunoprecipitated from the FAC fraction (Figure 9). Control reactions containing immunoprecipitates using nonimmune serum or lacking either FAC protein or enolase substrate were also negative (Figure 9). Background phosphorylation of IgG occurred in all reaction samples that contained antibody or serum, as previously observed (Clarke and Brugge, 1993). Thus, phosphorylation of enolase occurred only in the presence of immunoprecipitated pp60$^{c\text{-}src}$, confirming that the pp60$^{c\text{-}src}$ that is contained within the isolated FAC exhibits tyrosine kinase activity and that this activity

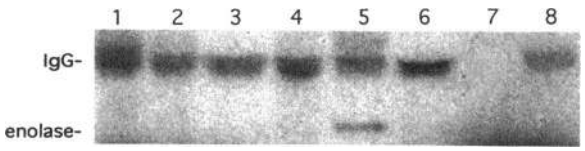

Figure 9. Isolated FACs are enriched for pp60$^{c\text{-}src}$ activity. pp60$^{c\text{-}src}$ was immuno-precipitated from whole cells cultured in the presence of soluble FN (50 μg/ml) for 30 min or from cells bound to RGD-beads at different stages in the FAC isolation procedure and subjected to an in vitro kinase assay using enolase as an exogenous substrate. Specific phosphorylation of the enolase band indicates pp60$^{c\text{-}src}$ activity. Lane 1, total cellular protein isolated from cells incubated with soluble FN (50 μg/ml) for 30 min in suspension; lane 2, total cellular protein isolated from cells incubated with RGD-beads for 30 min in suspension; lane 3, soluble proteins removed when bead-bound cells were extracted with detergent-containing buffer; lane 4, insoluble CSK proteins removed from the beads as a result of sonication and dounce homogenization; lane 5, the remaining bead-associated FAC. Controls, shown at the right, included the use of nonimmune serum in place of pp60$^{c\text{-}src}$ antibodies in conjunction with FAC proteins (lane 6) and carrying out the kinase reaction without either immunoprecipitate (lane 7) or enolase substrate (lane 8). Immunoprecipitations were carried out using equal protein samples; molecular weight standards for IgG and enolase are indicated at the left.

is greatly enriched when compared on an equal protein basis with either whole cells or the intact CSK.

We also found that isolated FACs contained pp125^{FAK} protein and that it remained active in vitro (Figure 10A). Densitometric quantitation of Western blots confirmed that pp125^{FAK} protein was enriched in the FAC by approximately 2- and 30-fold relative to the CSK and whole cell fractions, respectively (Figure 10B). This enrichment of pp125^{FAK} protein resulted in a corresponding twofold enhancement of pp125^{FAK} activity in the FAC compared with the intact CSK (Figure 10B).

We then examined whether isolated FACs retain inositol lipid kinase activities. Equal amounts of protein from the CSK, basal cell surface preparations, or isolated FAC fractions were incubated in vitro with [γ-^{32}P]ATP and exogenous PIP substrate; newly synthesized ^{32}P-labeled lipids were separated and analyzed by thin layer chromatography and autoradiography (Figure 11A). We have previously demonstrated that cell attachment to FN-coated dishes induces PIP_2 synthesis within minutes after cell binding (McNamee *et al.*, 1993). Densitometric quantitation of autoradiographs confirmed that isolated FACs were enriched approximately four- to fivefold for PIP kinase activity relative to the CSK and basal cell surface fractions, as indicated by an increase in the amount of radiolabeled PIP_2. PI-3-kinase activity should not be measureable in the detergent-containing buffer we utilize and thus, this activity most likely represents the activity of PI4P-5-kinase. However, as suggested by our immunofluorescence results (Figure 2), PI-3-kinase activity could be detected within isolated FACs

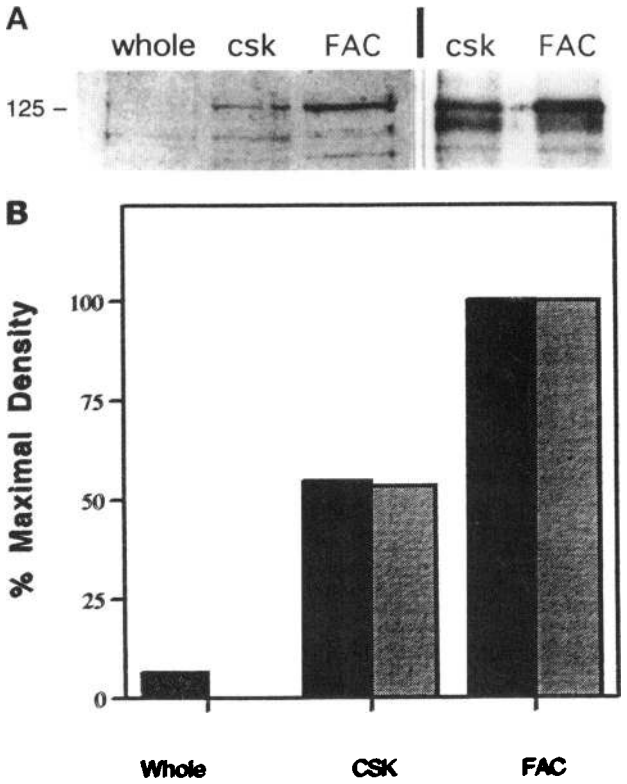

Figure 10. Isolated FACs were enriched for active pp125^{FAK}. (A) Left, Western blot of proteins isolated from whole cells (whole), intact CSK (csk), or isolated FACs (FAC) that were loaded on an equal protein basis and stained using antibodies against pp125^{FAK}. Right, pp125^{FAK} was immunoprecipitated from equal amounts of protein from the CSK and FAC fractions, subjected to an in vitro kinase (autophosphorylation) assay in the presence of [^{32}P]ATP, separated using SDS-PAGE, and analyzed by autoradiography. The molecular weight of the major protein species is indicated at the left. (B) Densitometric quantitation of the 125-KDa bands stained in the blots shown in panel A. Black bars, pp125^{FAK} protein; stippled bars, pp125^{FAK} activity.

when immunoprecipitated and analyzed free of detergent (Figure 11B). In contrast to the FAC-associated PI4P-5-kinase activity (Figure 11A), PI-3-kinase activity did not appear to be enriched within the FAC compared with the intact CSK fraction (Figure 11B). A direct comparison between PI-3-kinase and PI4P-5-kinase activities cannot be made because these kinases were not assayed in the same manner. However, these results are consistent with the finding that although both the 3,4 and 4,5 forms of PIP_2 are induced by cell binding to ECM, the 4,5 form is by far the dominant product (McNamee *et al.*, 1993).

Interestingly, a thin layer chromatography spot that co-migrated with PIP became labeled in the PIP kinase assay, even though exogenous PI was not included in the reaction (Figure 11A). A similar spot also became labeled when all exogenous lipid (i.e., PIP-containing liposomes) were omitted (Figure 11A). One possibility

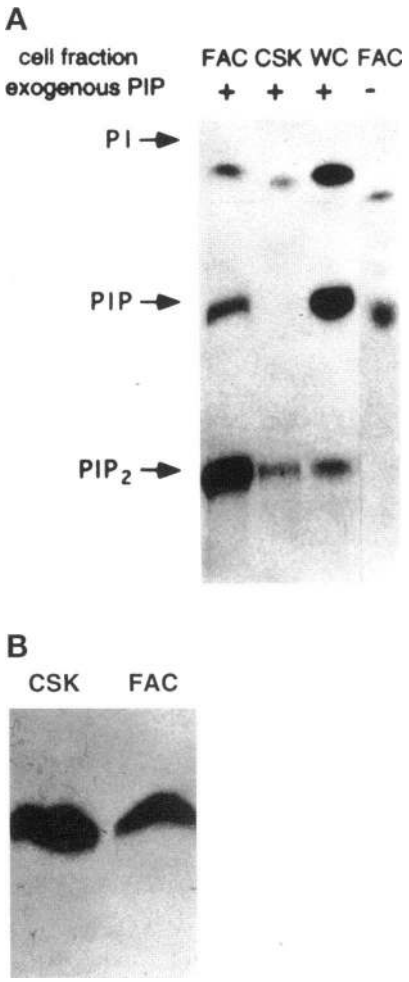

Figure 11. Isolated FACs exhibit multiple activities associated with the inositol lipid synthesis cascade. (A) PIP kinase activity was measured in vitro by incubating equal protein from FAC, CSK, and WC preparations with (+) or without (−) PI4P (PIP)-containing liposomes in the presence of [γ-^{32}P]ATP, separating the labeled products by thin layer chromatography, and visualizing them using autoradiography. Arrows indicate the position of PI, PIP, and PIP_2 standards. The radiolabeled spot that runs just below the PI standard exhibits the same mobility as a phosphatidic acid standard. (B) PI-3-kinase was immunoprecipitated from equal protein samples from intact CSK extracted from suspended cells bound to RGD-beads for 30 min (CSK) or from FACs that were isolated from these cells. PI-3-kinase activity was analyzed using PI as a substrate in an in vitro kinase assay after the immunoprecipitate was washed free of detergent. The single labeled spot comigrated with the PIP standard.

is that isolated FACs contain CSK-associated PI (i.e., even after extensive detergent washes) that retains its ability to act as a substrate for its normal downstream modifying enzyme PI kinase. The other spot that migrated slightly below PI in the presence and absence of exogenous substrate (Figure 11A) may be phosphatidic acid, the product of diacylglycerol kinase, because the same spot became labeled when diacylglycerol was used as an exogenous substrate (our unpublished observations). Taken together, these findings suggest that multiple active enzymes and substrates that mediate sequential steps in the inositol lipid signaling pathway, including PI, PI-3- and 4-kinases, and PI4P-5-kinase, remain tightly associated with the CSK framework of the FAC and remain functionally active after detergent extraction and magnetic isolation.

DISCUSSION

Many studies demonstrate that cell-ECM interactions are critical during cell growth and differentiation. Yet, the molecular mechanisms linking these interactions with specific cell functions remain largely unknown. We and others have demonstrated that integrin binding and clustering activate intracellular signalling pathways, suggesting that integrins act as cell surface signal transducers (Ingber, 1991; Damsky and Werb, 1992; Hynes, 1992; Schwartz and Ingber, 1994). However, because integrins possess no intrinsic kinase activity, they must somehow associate with downstream signaling molecules. Furthermore, although most studies have focused on tyrosine kinases, it is known that many other types of signaling molecules (e.g., PI kinases and ion channels) are involved in this response and that integrin-based signals rapidly integrate with those elicited by growth factor receptors. Thus, the fundamental question remains: how does integrin binding simultaneously activate these diverse signaling pathways and yet produce a single concerted cellular response?

The present results suggest a possible mechanism for signal integration that is based on cell structure rather than solution chemistry. It is well known that integrins induce formation of a specialized CSK complex or FAC at the site of cell-ECM binding due to specific binding interactions between integrins, actin-associated molecules, and actin (Burridge *et al.*, 1988). Our data suggest that this insoluble scaffolding, in turn, immobilizes cell surface growth factor receptors as well as numerous chemical signaling molecules and brings them into close proximity within the FAC. This type of "solid-state" signaling may facilitate cross-talk between multiple signaling pathways that normally function at many sites in the cell. This signaling scaffolding also should be exquisitely sensitive to mechanical stresses because the FAC provides a path for mechanical signal transfer (Wang *et al.*, 1993). Perhaps it is for this reason that cells can simultaneously process chemical and mechanical stimuli (Ingber, 1991; Schwartz and Ingber, 1994).

Past study of FAC function was hindered by the finding that formation of well organized and easily detectable FACs normally only occurs hours to days after cell plating (Truskey *et al.*, 1992). In contrast,

ECM-dependent activation of chemical signaling events occurs within minutes after integrin binding (Ingber *et al.*, 1990; Shattil and Brugge, 1991; Kornberg *et al.*, 1992; Lipfert *et al.*, 1992; Sorisky *et al.*, 1992; Cybulsky *et al.*, 1993; McNamee *et al.*, 1993). Thus, it has been extremely difficult to examine FAC assembly and function during the relevant time course when signaling events are first activated in response to integrin binding. This is likely why only a limited number of specific chemical signaling molecules have been previously localized within FACs, most notably, $pp125^{FAK}$, viral tyrosine kinases, and protein kinase C. More recently, methods have been developed to isolate subfractions of cells that are greatly enriched for FACs that remain adherent to culture surfaces after cells are removed (Niederreiter *et al.*, 1994). Although these methods permit compositional analysis, the amount of protein isolated is extremely low and thus, study of associated enzyme activities is significantly limited. More importantly, these FACs can only be isolated many hours after cell plating when well developed FACs are visible and thus, many hours after initial integrin signaling events are triggered.

We circumvented this problem in the present study by allowing cells to bind to magnetic microbeads that were coated with integrin ligands. These beads induced integrin clustering and FAC formation within 15 min after binding to capillary endothelial cells (Plopper and Ingber, 1993). Bead binding also nicely mimics cell binding to ECM-coated dishes in that it activates multiple chemical signaling pathways, including Na^+/H^+ exchange (Schwartz *et al.*, 1991), inositol lipid synthesis (McNamee and Ingber, 1993), and protein tyrosine phosphorylation (Figures 5, 9, and 10) over a similar time course. Importantly, bead-associated FACs can be physically isolated away from the remainder of the cell and CSK based on the magnetic nature of these beads (Plopper and Ingber, 1993). We have previously shown that these isolated complexes are specifically enriched for known FAC proteins, including $\beta 1$ integrin, talin, vinculin, α-actinin, and paxillin (Plopper and Ingber, 1993). These FACs also are not simply pieces of the cortical CSK because complexes that were isolated using the same beads coated with ligands for metabolic receptors (e.g., AcLDL; Figure 4) differed significantly in terms of their protein composition. In addition, we have recently found that complexes isolated from umbilical vein endothelial cells using the same magnetic beads coated with ligands for a different type of adhesion receptor (E-selectin) also differ in composition from FACs isolated using RGD-beads (talin and tensin are not present; Yoshida, Westlin, Wang, Ingber, Rozenzweig, Resnick, and Gimbrone, unpublished data). However, it is important to clarify that we have not optimized our FAC procedure to permit isolation of all cellular FAC proteins with 100% efficiency. In fact, the average cell binds only a few beads in our method and hence, only forms a small number of the many FACs that potentially can be produced. Nevertheless, this new approach offers the unique opportunity to analyze changes in the relative distribution and activity of chemical signaling molecules within the FAC during the time when these molecules are first activated in response to cell attachment to ECM. These early times are also when many signaling molecules redistribute to the CSK following stimulation by growth factors (Clarke and Brugge, 1993; Rankin and Rozengurt, 1994).

When we carried out immunolocalization experiments, protein tyrosine kinases, ion channels, and enzymes involved in inositol lipid metabolism all became associated with the CSK and concentrated within the FAC within minutes after integrin binding. All of these molecules remained associated with the insoluble FACs after isolation and were found to be enriched in this fraction when compared with either whole cells, intact CSK, or a basal cell surface preparation that retains plasma membrane. $pp125^{FAK}$ and $pp60^{v\text{-}src}$ have been previously shown to localize within FACs by immunostaining (Rohrschneider *et al.*, 1982; Schaller *et al.*, 1992) and the Na^+/H^+ antiporter was found to codistribute with vinculin, talin, and F-actin in a recent report that used a different source of antibodies (Grinstein *et al.*, 1993). Most importantly, we found the high affinity FGF receptor *flg* was not only present within the FAC, it was greatly enriched compared with either the whole cell or intact CSK. The possibility that FGF receptors concentrate within the FAC is supported by the recent finding that *flg* associates with $pp60^{c\text{-}src}$ and cortactin (Zhan *et al.*, 1994). Cortactin is a putative substrate for $pp60^{v\text{-}src}$ that contains F-actin binding and SH3 domains; it also has been reported to be concentrated in CSK structures that are enriched for FAC proteins (Wu *et al.*, 1991). Subsets of other functional active growth factor receptors (e.g., PDGF-β receptor) similarly become triton insoluble and sequestered within microdomains at the cell surface in other cell types (T. Daniels, Vanderbilt University, personal communication), although the exact location of these receptor binding sites is not known.

The tyrosine kinase $pp125^{FAK}$, which is both a major FAC component and a substrate for $pp60^{v\text{-}src}$ (Guan and Shalloway, 1992), has been previously shown to represent a point of convergence between growth factor and ECM-dependent signaling mechanisms (Zachary and Rozengurt, 1992). Importantly, both of these kinases were rapidly and specifically recruited to the cell-bead interface when cells bound to RGD-beads, and this change in distribution correlated with an increase in PTyr staining in the same region. These morphological results were confirmed by biochemical studies that demonstrated that bead-associated FACs

were enriched for PTyr-containing proteins as well as both $pp60^{c\text{-}src}$ and $pp125^{FAK}$ activities after magnetic isolation when compared with either whole cells or intact CSK. One caveat in these studies is that the antibodies we used to analyze $pp60^{c\text{-}src}$ are polyclonal antibodies that were raised against src gene product. We cannot rule out the possibility that these antibodies cross-react with other src-related kinases that exhibit similar activities and molecular weight. However, this would not significantly change the general interpretation of our results, namely, that integrin binding results in relative enrichment and immobilization of active tyrosine kinases on the CSK backbone of the FAC. In addition, our results suggest that isolated FACs may also contain active nontyrosine kinases (Figure 8). The identities of these enzymes remain to be defined, however, at least one serine/threonine kinase, protein kinase C, is known to be activated by integrin binding (Vuori and Ruoslahti, 1993) and to localize to the FAC (Jaken *et al.*, 1989).

In addition, isolated FACs were found to retain activities that are necessary to progress through multiple sequential steps in the inositol signaling cascade (e.g., PI to PIP to PIP_2). This in vitro signaling capacity appeared to be based on retention of multiple enzymes that are involved in the inositol lipid synthesis cascade (e.g., PI-3- and 4-kinases, PI-4P5-kinase) as well as CSK-associated lipid substrates (e.g., PI). It is important to emphasize that downstream elements that are involved in the degradative portion of the inositol signaling pathway, such as PLC and the Na^+/H^+ antiporter, are also retained within the same complex.

Taken together, these results suggest that the FAC that forms within minutes following cell-ECM binding may represent a major site for chemical signal processing inside the cell. In addition, our data imply that much of "membrane signaling" may effectively proceed in a "solid-state" along insoluble CSK scaffolds. In this context, solid-state refers to a regulatory arrangement in which multiple steps in a biochemical cascade are spatially integrated and coordinated by immobilizing the relevant enzymes and substrates and placing them in close proximity. This mechanism for signaling is likely not limited to endothelial cells because the membrane skeleton similarly mediates binding of signaling molecules at sites of integrin-CSK interactions in platelets (Fox *et al.*, 1993). Many other metabolic pathways, including protein synthesis, glycolysis, RNA processing, and DNA synthesis, also appear to use a solid-state mechanism to channel biochemical reactions and hence, to increase the efficiency of cell function (Ingber, 1993). Importantly, in this type of regulatory scheme, a new target for control of signal transduction emerges: spatial positioning within the cell. If the CSK regulates the location of chemical signaling molecules, then we would predict that these molecules should possess specific CSK binding domains or binding sites for docking proteins that would, in turn, mediate CSK linkages. In fact, SH3 domains can mediate associations between signaling molecules and CSK elements (Koch *et al.*, 1991; Bar-Sagi *et al.*, 1993). Binding of PTyr residues to SH2 domains within bifunctional proteins, such as tensin (Davis *et al.*, 1991) and $pp125^{FAK}$ (Schlaepfer *et al.*, 1994) also may lead to indirect CSK associations.

This coalescence of receptors for growth factors (*flg*) and ECM (integrin β_1; Plopper and Ingber, 1993) as well as functionally active signaling molecules within a localized microdomain at the cell surface (i.e., the FAC) may, in part, explain how signals from these two different types of receptors can be rapidly integrated at the cell surface (e.g., during activation of the Na^+/H^+ antiporter in capillary endothelial cells; Ingber *et al.*, 1990). It also may help to explain previously cryptic results. For example, the codistribution of synthetic (PI kinase and PIP kinases) and degradative (PLC) elements in the inositol lipid signaling cascade within the tight confines of the FAC provides a possible explanation for how local integrin-dependent synthesis of PIP_2 can be rate-limiting for growth factor-stimulated breakdown of this inositol lipid by PLC (McNamee *et al.*, 1993). The possibility that a local PIP_2 pool exists, immobilized on the CSK within the FAC, is consistent with the finding that PIP_2 binds CSK proteins with high affinity (Janmey and Stossel, 1987; Goldschmidt-Clermont *et al.*, 1990) and that certain other membrane lipids form microdomains and do not appear to be freely mobile in the plasma membrane (Edidin, 1993). Similarly, enrichment of the Na^+/H^+ antiporter within the FAC raises the possibility that increased antiporter activity, due to stimulation by growth factors and cell binding to ECM, may alter downstream signaling events and influence cell growth by producing local changes in proton concentration (and pH) directly within the interstices of the densely packed CSK framework of the FAC upon which many signaling molecules are immobilized. This would explain why the gross measurement of cytoplasmic pH does not consistently correlate with growth, although interfering with antiporter activation can inhibit cell proliferation (Ingber *et al.*, 1990).

In this manner, a single cellular response may result from establishment of structural arrangements within the CSK that cause signaling components from different transduction pathways to codistribute locally and hence, cross-talk at the site of integrin binding. However, although FACs provide an insoluble CSK scaffolding for organizing chemical signaling molecules, they are also very dynamic structures. For example, FACs have been shown to remodel over a period of minutes in living endothelial cell monolayers (Davies *et al.*, 1994) and their structural integrity appears to be highly sensitive to changes in signal transduction

(Jaken *et al.*, 1989; Rahilly and Fleming, 1992; Ridley and Hall, 1992; Woods and Couchman, 1992). This dynamic nature of the FAC and its relation to signal transduction mechanisms involved in growth control may explain why certain signaling molecules have been observed to localize to FACs in certain cells and not in others.

In conclusion, our data suggest that positioning of growth factor receptors, integrins, and chemical signaling molecules along CSK scaffolds within the FAC may serve to immediately integrate cues from soluble mitogens with those resulting from cell binding to ECM and transmission of mechanical stresses across the plasma membrane. This form of spatial integration at the cell surface may, in part, explain how cells can sense multiple simultaneous inputs and yet produce a single, concerted response. As a highly organized subcellular structure of unique molecular composition that performs specialized cell functions (e.g., anchorage, chemical and mechanical signal integration), it may be useful to think of the FAC as an organelle even though it is not membrane bound. As in the case of "classical" organelles, development of a method to isolate these structures in a form amenable to biochemical analysis (as accomplished in this study) should help to decipher FAC function and further characterize its molecular composition. This new approach that permits analysis of chemical signaling in a structurally relevant context could also facilitate development of new therapeutics (e.g., angiogenesis inhibitors) that target interactions between signaling molecules and the CSK, rather than the signaling molecules themselves.

ACKNOWLEDGMENTS

We thank Drs. Tom Maciag, Martin Schwartz, Tom Parsons, Tom Roberts, Morris White, and Igor Prudovsky for kindly supplying antibodies and for their helpful suggestions. This work was supported by grants from the United States Army Medical Research and Materiel Command (DAMD17-94-J-4283) and National Institutes of Health (HL-46491). H.M. is a recipient of a student fellowship from NASA. D.I. is a recipient of a Faculty Research Award from the American Cancer Society.

REFERENCES

Banga, H.S., Simons, E.R., Brass, L.F., and Rittenhouse, S.E. (1986). Activation of phospholipases A and C in human platelets exposed to epinephrine: role of glycoproteins IIb/IIIa and dual role of epinephrine. Proc. Natl. Acad. Sci. USA *83*, 9197–9201.

Ball, E.H., Freitag, C., and Gurofsky, S. (1986). Vinculin interaction with permeabilized cells: disruption and reconstitution of a binding site. J. Cell Biol. *10*, 641–648.

Bar-Sagi, D., Rotin, D., Batzer, A., Mandiyan, V., and Schlessinger, J. (1993). SH3 domains direct cellular localization of signaling molecules. Cell *74*, 83–91.

Beckerle, M.C., Burridge, K., DeMartino, G.N., and Croall, D.E. (1987). Colocalization of calcium-dependent protease II and one of its substrates at sites of cell adhesion. Cell *51*, 569–577.

Brands, R., and Feltkamp, C.A. (1988). Wet cleaving of cells: a method to introduce macromolecules into the cytoplasm. Exp. Cell Res. *176*, 309–318.

Brooks, P.C., Montgomery, A.M.P., Rosenfeld, M., Reisfeld, R.A., Hu, T., Klier, G., and Cheresh, D.A. (1994). Integrin $\alpha v\beta 3$ antagonists promote tumor regression by inducing apoptosis of angiogenic blood vessels. Cell *79*, 1157–1164.

Burr, J.G., Dreyfuss, G., Penman, S., and Buchanan, J.M. (1980). Association of the src gene product of Rous sarcoma virus with cytoskeletal structures of chicken embryo fibroblasts. Proc. Natl. Acad. Sci. USA *77*, 3484–3488.

Burridge, K., Fath, K., Kelly, T., Nuckolls, G., and Turner, C. (1988). Focal adhesions: transmembrane junctions between the extracellular matrix and the cytoskeleton. Annu. Rev. Cell Biol. *4*, 487–525.

Chen, Q., Kinch, M.S., Lin, T.H., Burridge, K., and Juliano, R.L. (1994). Integrin-mediated cell adhesion activates mitogen-activated protein kinases. J. Biol. Chem. *269*, 26602–26605.

Chun, J.S., and Jacobson, B.S. (1992). Requirement for diacylglycerol and protein kinase C in HELA cell-substratum adhesion and their feedback amplification of arachidonic acid production for optimum cell spreading. Mol. Biol. Cell *4*, 271–281.

Clark, E.A., and Brugge, J.S. (1993). Redistribution of activated pp60c-src to integrin-dependent cytoskeletal complexes in thrombin-stimulated platelets. Mol. Cell. Biol. *13*, 1863–1871.

Cobb, B., Schaller, M., Leu, T.-H., and Parsons, T.H. (1994). Stable association of $pp60^{src}$ and $pp59^{fyn}$ with the focal adhesion-associated protein tyrosine kinase $pp25^{FAK}$. Mol. Cell. Biol. *14*, 147–155.

Cooper, J.A., and Hunter, T. (1981). Changes in protein phosphorylation in Rous sarcoma virus-transformed chicken embryo cells. Mol. Cell. Biol. *1*, 165–178.

Cybulsky, A.V., Carbonetto, S., Cyr, M.D., McTavish, A.J., and Huang, Q. (1993). Extracellular matrix-stimulated phospholipase activation is mediated by beta 1-integrin. Am. J. Physiol. *264*, C323–C332.

Damsky, C.H., and Werb, Z. (1992). Signal transduction by integrin receptors for extracellular matrix: cooperative processing of extracellular information. Curr. Opin. Cell Biol. *4*, 772–781.

Davies, P.F., Robotewskyj, A., and Griem, M.L. (1994). Quantitative studies of endothelial cell adhesion: directional remodeling of focal adhesion sites in response to flow forces. J. Clin. Invest. *93*, 2031–2038.

Davis, C.M., Danehower, S.C., Laurenza, A., and Molony, J.L. (1993). Identification of a role of the vitronectin receptor and protein kinase C in the induction of endothelial cell vascular formation. J. Cell. Biochem. *51*, 206–218.

Davis, S., Lu, M.L., Lo, S.H., Lin, S., Butler, J.A., Druker, B.J., Roberts, T.M., An, Q., and Chen, L.B. (1991). Presence of an SH2 domain in the actin-binding protein tensin. Science *252*, 712–715.

Edidin, M. (1993). Patches and fences: probing for plasma membrane domains. J. Cell Sci. *17*(suppl), 165–169.

Fey, E.G., Wan, K.M., and Penman, S. (1984). Epithelial cytoskeletal framework and nuclear matrix-intermediate filament scaffold: three dimensional organization and protein composition. J. Cell Biol. *98*, 1973–1984.

Fox, J.E.B., Lipfert, L., Clark, E.A., Reynolds, C.C., Austin, C.D., and Brugge, J.S. (1993). On the role of the platelet membrane skeleton in mediating signal transduction. J. Biol. Chem. *268*, 25973–25984.

Goldschmidt-Clermont, P.J., Machesky, L.M., Baldassare, J.J., and Pollard, T.D. (1990). The actin-binding protein profilin binds to PIP_2 and inhibits its hydrolysis by phospholipase C. Science *247*, 1575–1578.

Grinstein, S., Woodside, M., Waddell, T.K., Downey, G.P., Orlowski, J., Pouyssegur, J., Wong, D.C., and Foskett, J.K. (1993). Focal localization of the NHE-1 isoform of the Na^+/H^+ antiport: assessment of effects on intracellular pH. EMBO J. *12*, 5209–5218.

Guan, J.L., and Shalloway, D. (1992). Regulation of focal adhesion-associated protein tyrosine kinase by both cellular adhesion and oncogenic transformation. Nature *358*, 690–692.

Guan, J.L., Trevithick, J.E., and Hynes, R.O. (1991). Fibronectin/integrin interaction induces tyrosine phosphorylation of a 120-kDa protein. Cell Regul. *2*, 951–964.

Hansen L., Mooney, D., Vacanti, J.P., and Ingber, D.E. (1994). Integrin binding and cell spreading on extracellular matrix act at different points in the cell cycle to promote hepatocyte growth. Mol. Biol. Cell *5*, 967–975.

Hildebrand, J.D., Schaller, M.D., and Parsons, J.T. (1993). Identification of sequences required for the efficient localization of the focal adhesion kinase, pp125FAK, to cellular focal adhesions. J. Cell Biol. *123*, 993-1005.

Hynes, R. (1992). Integrins: versatility, modulation, and signaling in cell adhesion. Cell *69*, 11–25.

Ingber, D.E. (1990). Fibronectin controls capillary endothelial cell growth by modulating cell shape. Proc. Natl. Acad. Sci. USA *87*, 3579–3583.

Ingber, D.E. (1991). Integrins as mechanochemical transducers. Curr. Opin. Cell Biol. *3*, 841–848.

Ingber, D.E. (1992). Extracellular matrix as a solid state regulator of angiogenesis: identification of new targets for anti-cancer therapy. Semin. Cancer Biol. *3*, 57–63.

Ingber, D.E. (1993). The riddle of morphogenesis: a question of solution chemistry or molecular cell engineering? Cell *75*, 1249–1252.

Ingber, D.E., and Folkman, J. (1989a). How does extracellular matrix control capillary morphogenesis? Cell *58*, 803–805.

Ingber, D.E., and Folkman, J. (1989b). Mechanochemical switching between growth and differentiation during fibroblast growth factor-stimulated angiogenesis in vitro: role of extracellular matrix. J. Cell Biol. *109*, 317–330.

Ingber, D.E., Madri, J.A., and Folkman, J. (1986). A possible mechanism for inhibition of angiogenesis by angiostatic steroids: induction of capillary basement membrane dissolution. Endocrinology *119*, 1768–1775.

Ingber, D.E., Prusty, D., Frangione, J., Cragoe, E.J., Jr., Lechene, C., and Schwartz, M. (1990). Control of intracellular pH and growth by fibronectin in capillary endothelial cells. J. Cell Biol. *110*, 1803–1812.

Ingber, D.E., Prusty, D., Sun, Z., Betensky, H., and Wang, N. (1995). Cell shape, cytoskeletal mechanics and cell cycle control in angiogenesis. J. Biomech. (*in press*).

Jaken, S., Leach, K., and Klauck, T. (1989). Association of type 3 protein kinase C with focal contacts in rat embryo fibroblasts. J. Cell Biol. *109*, 697–704.

Janmey, P.A., and Stossel, T.P. (1987). Modulation of gelsolin functions by phosphatidylinositol-4,5-biphosphate. Nature *325*, 362–364.

Kapron-Bras, C., Fitz-Gibbon, L., Jeevaratnam, P., Wilkins, J., and Dedhar, S. (1993). Stimulation of tyrosine phosphorylation and accumulation of GTP-bound $p21^{ras}$ upon antibody-mediated $\alpha 2\beta 1$ integrin activation in T-lymphoblastic cells. J. Biol. Chem. *268*, 20701–20704.

Kellie, S., Patel, B., Mitchell, A., Critchley, D.R., Wigglesworth, N.M., and Wyke, J.A. (1986a). Comparison of the relative importance of tyrosine-specific vinculin phosphorylation and the loss of surface-associated fibronectin in the morphology of cells transformed by Rous sarcoma virus. J. Cell Sci. *82*, 129–142.

Kellie, S., Patel, B., Wigglesworth, N.M., Critchley, D.R., and Wyke, J.A. (1986b). The use of Rous sarcoma virus transformation mutants with differing tyrosine kinase activities to study the relationships between vinculin phosphorylation, $pp60^{v\text{-}src}$ location and adhesion plaque integrity. Exp. Cell Res. *165*, 216–228.

Koch, C.A., Anderson, D., Moran, M.F., Ellis, C., and Pawson, T. (1991). SH2 and SH3 domains: elements that control interactions of cytoplasmic signaling proteins. Science *252*, 668–674.

Kornberg, L., Earp, H.S., Parsons, J.T., Schaller, M., and Juliano, R.L. (1992). Cell adhesion or integrin clustering increases phosphorylation of a focal adhesion-associated tyrosine kinase. J. Biol. Chem. *267*, 23439–23442.

Kume, N., Arai, H., Kawai, C., and Kita, T. (1991). Receptors for modified low-density lipoproteins on human endothelial cells: different recognition for acetylated low-density lipoprotein and oxidized low-density lipoprotein. Biochim. Biophys. Acta *1091*, 63–67.

Lewis, J.M., and Schwartz, M.A. (1995). Mapping in vivo associations of cytoplasmic proteins with integrin $\beta 1$ cytoplasmic domain mutants. Mol. Biol. Cell *6*, 151–160.

Ling, L.E., Schultz, J.T., and Cantley, L.C. (1989). Characterization and purification of membrane-associated phosphatidylinositol-4-phosphate kinase from human red blood cells. J. Biol. Chem. *264*, 5080–5088.

Lipfert, L., Haimovich, B., Schaller, M.D., Cobb, B.S., Parsons, J.T., and Brugge, J.S. (1992). Integrin-dependent phosphorylation and activation of the protein tyrosine kinase $pp125^{FAK}$ in platelets. J. Cell Biol. *119*, 905–912.

Maher, P.A., Pasquale, E.B., Wang, J.Y., and Singer, S.J. (1985). Phosphotyrosine-containing proteins are concentrated in focal adhesions and intercellular junctions in normal cells. Proc. Natl. Acad. Sci. USA *82*, 6576–6580.

Marchisio, P.C., Di Renzo, M.F., and Comoglio, P.M. (1984). Immunofluorescence localization of phosphotyrosine-containing proteins in RSV-transformed mouse fibroblasts. Exp. Cell Res. *154*, 112–124.

Masters, D.B., Griggs, C.T., and Berde, C.B. (1992). High sensitivity quantification of RNA from gel and autoradiograms with affordable optical scanning. Biotechniques *12*, 902–911.

McNamee, H.P., and Ingber, D.E. (1993). Integrin-dependent activation of phosphoinositide kinases occurs within the focal adhesion complex. Mol. Biol. Cell 4, 362a.

McNamee, H.P., Ingber, D.E., and Schwartz, M.A. (1993). Adhesion to fibronectin stimulates inositol lipid synthesis and enhances PDGF-induced inositol lipid breakdown. J. Cell Biol. *121*, 673–678.

Meisenhelder, J., and Hunter, T. (1992). The SH2/SH3 domain-containing protein Nck is recognized by certain anti-phospholipase C-gamma 1 monoclonal antibodies, and its phosphorylation on tyrosine is stimulated by platelet-derived growth factor and epidermal growth factor treatment. Mol. Cell. Biol. *12*, 5843–5856.

Miyamoto, S., Akiyama, S.K., and Yamada, K.M. (1995). Synergistic roles for receptor occupancy and aggregation in integrin transmembrane function. Science *267*, 883–885.

Niederreiter, M., Gimona, M., Streichsbier, F., Celis, J.E., and Small, J.V. (1994). Complex protein composition of isolated focal adhesions: a two-dimensional gel and database analysis. Electrophoresis *15*, 511–519.

Oda, A., Druker, B.J., Ariyoshi, H., Smith, M., and Salzman, E.W. (1993). $pp60^{src}$ is an endogenous substrate for calpain in human blood platelets. J Biol. Chem. *268*, 12603–12608.

Pelletier, A.J., Bodary, S.C., and Levinson, A.D. (1992). Signal transduction by the platelet integrin alpha IIb beta 3: induction of cal-

cium oscillations required for protein-tyrosine phosphorylation and ligand-induced spreading of stably transformed cells. Mol. Biol. Cell *3*, 989–998.

Plopper, G., and Ingber, DE. (1993). Rapid induction and isolation of focal adhesion complexes. Biochem. Biophys. Res. Commun. *193*, 571–578.

Rahilly, M.A., and Fleming, S. (1992). A tumor promoter induces alterations in vinculin and actin distribution in human renal epithelium. J. Pathol. *166*, 283–288.

Rankin, S., and Rozengurt, E. (1994). Platelet-derived growth factor modulation of focal adhesion kinase (p125FAK) and paxillin tyrosine phosphorylation in Swiss 3T3 cells. J. Biol. Chem. *269*, 704–710.

Ridley, A.J., and Hall, A. (1992). The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. Cell *70*, 389–399.

Rohrschneider, L., Rosok, M., and Shriver, K. (1982). Mechanism of transformation by Rous sarcoma virus: events within adhesion plaques. Cold Spring Harb. Symp. Quant. Biol. *46*, 953–965.

Ross, W., Bertrand, W., and Morrison, A. (1990). A photoactivatable probe for the Na^+/H^+ exchanger cross-links a 66-kDa renal brush border membrane protein. J. Biol. Chem. *265*, 5341–5344.

Sardet, C., Counillon, L., Franchi, A., and Pouyssegur, J. (1990). Growth factors induce phosphorylation of the Na^+/H^+ antiporter, glycoprotein of 110 kD. Science *247*, 723–726.

Schaller, M.D., Borgman, C.A., Cobb, B.S., Vines, R.R., Reynolds, A.B., and Parsons, J.T. (1992). $pp125^{FAK}$ a structurally distinctive protein-tyrosine kinase associated with focal adhesions. Proc. Natl. Acad. Sci. USA *89*, 5192–5196.

Schlaepfer, D.D., Hanks, S.K., Hunter, T., and van der Geer, P. (1994). Integrin-mediated signal transduction linked to Ras pathway by GRB2 binding to focal adhesion kinase. Nature *372*, 786–789.

Schwartz, M.A. (1993). Spreading of human endothelial cells on fibronectin or vitronectin triggers elevation of intracellular free calcium. J. Cell Biol. *120*, 1003–1010.

Schwartz, M.A., and Ingber, D.E. (1994). Integrating with integrins. Mol. Biol. Cell *5*, 389–393.

Schwartz, M.A., Lechene, C., and Ingber, D.E. (1991). Insoluble fibronectin activates the Na^+/H^+ antiporter by clustering and immobilizing integrin alpha 5 beta 1, independent of cell shape. Proc. Natl. Acad. Sci. USA *88*, 7849–7853.

Shattil, S.J., and Brugge, J.S. (1991). Protein tyrosine phosphorylation and the adhesive functions of platelets. Curr. Opin. Cell Biol. *3*, 868–879.

Sorisky, A., King, W.G., and Rittenhouse, S.E. (1992). Accumulation of $PtdIns(3,4)P_2$ and $PtdIns(3,4,5)P_3$ in thrombin-stimulated platelets: different sensitivities to Ca^{2+} or functional integrin. Biochem. J. *286*, 581–584.

Symington, B.E. (1992). Fibronectin receptor modulates cyclin-dependent kinase activity. J. Biol. Chem. *267*, 25744–25747.

Tanaka, A., Gibbs, C., Arthur, R.R., Anderson, S.K., Kung, H.-J., and Fujita, D.J. (1987). DNA sequence encoding the NH2-terminal region of the human c-src protein: implications of sequence divergence among src-type kinase oncogenes. Mol. Cell. Biol. *7*, 1978–1983.

Tapley, P., Horwitz, A.F., Buck, C.A., Burridge, K., Duggan, K., Hirst, R., and Rohrschneider, L. (1989). Analysis of the avian fibronectin receptor (integrin) as a direct substrate for $pp60^{v\text{-}src}$. Oncogene *4*, 325–333.

Torti, M., Sinigaglia, F., Ramaschi, G., and Balduini, C. (1991). Platelet glycoprotein IIb-IIIa is associated with 21-kDa GTP-binding protein. Biochim. Biophys. Acta *1070*, 20–26.

Truskey, G.A., Burmeister, J.S., Grapa, E., and Reichert, W.M. (1992). Total internal reflection fluorescence microscopy (TIRFM). II. Topographical mapping of relative cell/substratum separation distances. J. Cell Sci. *103*, 491–499.

Turner, C.E., Pavalko, F.M., and Burridge, K. (1989). The role of phosphorylation and limited proteolytic cleavage of talin and vinculin in the disruption of focal adhesion integrity. J. Biol. Chem. *264*, 11938–11944.

Vuori, K., and Ruoslahti, E. (1993). Activation of protein kinase C precedes $\alpha5\beta1$ integrin-mediated cell spreading on fibronectin. J. Biol. Chem. *268*, 21459–21462.

Wang, N., Butler, J.P., and Ingber, D.E. (1993). Mechanotransduction across the cell surface and through the cytoskeleton. Science *260*, 1124–1127.

Wang, N., and Ingber, D.E. (1994). Control of cytoskeletal mechanics by extracellular matrix, cell shape, and mechanical tension. Biophys. J. *66*, 2181–2189.

Woods, A., and Couchman, J.R. (1992). Protein kinase C involvement in focal adhesion formation. J. Cell Sci. *101*, 277–290.

Wu, H., Reynolds, A.B., Kanner, S.B., Vines, R.R., and Parsons, J.T. (1991). Identification and characterization of a novel cytoskeleton-associated $pp60^{src}$ substrate. Mol. Cell. Biol. *11*, 5113–5124.

Xing, Z., Chen, H.-C., Nowlen, J.K., Taylor, S.J., Shalloway, D., and Guan, J.-L. (1994). Direct interaction of v-src with the focal adhesion kinase mediated by the src SH2 domain. Mol. Biol. Cell *5*, 413–421.

Zachary, I., and Rozengurt, E. (1992). Focal adhesion kinase ($p125^{FAK}$): a point of convergence in the action of neuropeptides, integrins and oncogenes. Cell *71*, 891–894.

Zhan, X., Hu, X., Friesel, R., and Maciag, T. (1993). Long term growth factor exposure and differential tyrosine phosphorylation are required for DNA synthesis in BALB/c 3T3 cells. J. Biol. Chem. *268*, 9611–9620.

Zhan, X., Plourde, C., Hu, X., Friesel, R., and Maciag, T. (1994). Association of fibroblast growth factor receptor-1 with c-src correlates with association between c-src and cortactin. J. Biol. Chem. *269*, 1–4.