Integrin-Dependent Control of Inositol Lipid Synthesis in Vascular Endothelial Cells and Smooth Muscle Cells

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Extracellular matrix (ECM) molecules, such as fibronectin (FN), regulate fibroblast sensitivity to soluble growth factors, in part, by controlling cellular levels of phosphatidylinositol bis-phosphate (PIP_2), the substrate for phospholipase C-γ (McNamee *et al.,* 1993, *J. Cell Biol.* 121, 673–678). In the present study, we extended these investigations by exploring whether cells of the vascular wall also exhibit this response and analyzing the mechanism by which adhesion to ECM regulates intracellular PIP_2 mass. Capillary endothelial cells, pulmonary vascular smooth muscle cells, and C3H 101/2 fibroblasts were all found to exhibit a similar two- to threefold increase in PIP_2 mass within 3 h after binding to dishes coated with FN. Furthermore, similar effects were observed using dishes coated with a variety of different ECM molecules, including collagen types I and IV as well as a synthetic RGD-containing peptide. An increase in PIP_2 mass also was produced when suspended cells bound to microbeads (4.5 μm diameter; coated with RGD-peptide or anti-integrin β_1 antibody) that induce local integrin clustering and focal adhesion formation, independently of cell spreading. In contrast, neither binding of soluble FN nor binding of microbeads coated with ligands for other transmembrane surface receptors (e.g., acetylated low-density lipoprotein, antibodies against heparan sulfate) had any effect on PIP_2 mass. While these results suggest that integrin clustering stimulates PIP_2 synthesis, no change in total cellular or cytoskeletal-associated phosphatidylinositol-4-phosphate kinase (PIP kinase) activity could be detected when cells bound to immobilized integrin ligands. However, when focal adhesion complexes were isolated from these cells using a magnetic procedure (G. Plopper and D. E. Ingber, 1993, *Biochem. Biophys. Res. Commun.* 193, 571–578), this subfraction of the cytoskeleton was found to be enriched for PIP kinase activity by more than twofold relative to the whole cytoskeleton. These data suggest that ECM binding may increase PIP_2 mass in vascular cells by clustering cell surface integrin receptors and activating cytoskeletal-associated PIP kinases locally within the focal adhesion complex. © 1996 Academic Press, Inc.

INTRODUCTION

Cells attach to extracellular matrix (ECM) through binding of transmembrane receptors on their surfaces, such as integrins [1]. Integrin ligation and clustering promote formation of a specialized cytoskeletal structure that is known as the focal adhesion complex (FAC). FACs contain actin-associated proteins (e.g., talin, vinculin, paxillin, α-actinin) which physically interlink ECM, integrins, and cytoskeletal microfilaments and thereby, mechanically anchor cells to the substratum [2, 3]. In addition, integrin binding also activates intracellular signal transduction pathways that mediate many of the known effects of ECM on cell growth and differentiation [1, 4, 5]. However, little is known about how ECM controls these signaling events.

Previous work in this laboratory showed that inositol phosphate turnover is regulated by cell–ECM binding in C3H 10T1/2 fibroblasts [6]. In these cells, PDGF-induced release of inositol phosphates from phosphatidylinositol bis-phosphate (PIP_2) was suppressed when cells were suspended free of contact with ECM, even though PLC-γ (PLC) continued to be activated (tyrosine-phosphorylated) by PDGF. Total cellular PIP_2 mass decreased rapidly when cells were placed in suspension and reattachment to FN resulted in a rapid (within 2 min) increase in PIP_2 synthesis as well as a concomitant rise in total cellular PIP_2 mass. In other words, cell binding to FN appeared to control growth factor signaling and cell function by regulating the availability of PIP_2, the substrate for PLC. This model is supported by the finding that microinjection of constitutively activated RhoA, a low-molecular-weight G protein that stimulates PIP kinase activity, can circumvent the requirement for ECM binding and allow a calcium response to PDGF in suspended cells whereas inhibition of Rho processing using lovastatin both decreases total PIP_2 mass per cell and prevents the calcium response in adherent cells [7].

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Our laboratory is primarily interested in regulation of blood vessel development. While fibroblasts are an excellent *in vitro* model for biochemical studies on integrin signaling, the relevance of results obtained with these cells for vascular physiology remains unclear. Thus, in the present study, we first investigated whether regulation of PIP_2 mass by ECM is also exhibited by the major cellular components of the vessel wall: capillary endothelial (CE) cells and vascular smooth muscle (VSM) cells. We also analyzed the molecular mechanism by which cell binding to ECM induces inositol lipid synthesis in these cells.

MATERIALS AND METHODS

Cell lines. Bovine CE cells isolated from adrenal cortex were maintained at 10% CO_2 in low glucose Dulbecco's modified Eagle's medium (DMEM; 1000 mg/liter) supplemented with 10% calf serum, 10 μg/ml endothelial mitogen (Biomedical Technologies Inc.), and 20 mM Hepes, pH 7.4 [8]. CE cells were serum-deprived in DMEM with 1% calf serum without mitogen for 2 days before experiments. VSM cells were isolated from the main pulmonary artery of newborn calves [9] and maintained at 5% CO_2 in low glucose DMEM supplemented with 10% fetal calf serum and 20 mM Hepes, pH 7.4. Cells were used between the third and sixth passages. VSM cells were serum-deprived in DMEM with 0.5% fetal calf serum for 2 days before an experiment. C3H 10T1/2 cells were maintained at 5% CO_2 in high glucose (4500 mg/liter) DMEM containing 10% fetal calf serum [10]. Prior to plating for experiments, CE and 10T1/2 cells were trypsinized and then washed twice in appropriate DMEM containing 1% bovine serum albumin and 20 mM Hepes. VSM cells were pretreated with collagenase (10 mg/ml) in DMEM for 20 min before trypsinization. To focus on effects induced specifically by cell attachment to ECM, cells were cultured in chemically defined medium (DMEM/ 1% bovine serum albumin, 20 mM Hepes, 10 μg/ml high-density lipoprotein, 5 μg/ml transferrin) without exogenous peptide growth factor in all experiments. All culture media also were supplemented with fresh glutamine (0.3 mg/ml), penicillin (100 units/ml), streptomycin (100 μg/ml).

ECM coating procedures. The synthetic RGD peptide used was Peptite 2000 from Telios. Acetylated low-density lipoprotein (AcLDL) was obtained from Biotechnologies Inc., human FN from Cappel/ Organon Technica, and bovine collagen I and human collagen IV from Collaborative Research. All polypeptides were coated overnight at 4°C onto 100-mm bacteriological plastic dishes (Falcon) in 50 mM carbonate buffer, pH 9.4 (8 μg/ml; 10 ml/dish; approximately 1 μg protein/cm^2) to provide a saturating density of immobilized ligand [8]. We have previously shown that RGD peptide and FN produce similar effects on cell attachment, spreading, and growth when equal amounts of these adhesion molecules are preadsorbed onto bacteriological dishes [8, 11], even though the molar amount of RGD peptide (2 kDa) added exceeds that of FN approximately 100-fold. Microbeads were coated with proteins by incubating tosyl-activated magnetic microbeads (2×10^7/ml; Dynal) with proteins in carbonate buffer overnight at 4°C [12]. RGD peptides and AcLDL were added to beads at 50 μg/ml; antibodies against the extracellular domain of β1 integrin (clone B-D15; Biosource) and heparan sulfate glycosaminoglycan chains (clone HepSS-1; Seikagaku Corp.) were coated at 5 μg/ml. Any additional binding sites on the beads and dishes were blocked by incubating them with DMEM containing 1% BSA at 37°C prior to use. Nonadhesive dishes were prepared for CE and VSM cells by incubating noncoated petri dishes with DMEM containing 1% BSA and for C3H 10T1/2 cells by coating dishes with the nonadhesive polymer, poly-hydroxyethylmethacrylate [6, 13].

Quantitation of PIP_2 mass. In experiments analyzing the effects of cell adhesion on PIP_2 mass, cells were either plated onto ECM-coated dishes or maintained in suspension on nonadhesive dishes for 3 h at 37°C. In experiments in which cell binding to microbeads was studied, cells were placed in suspension with or without coated beads (20 beads/cell) in 1.5-ml tubes and maintained on an inverting platform at 37°C for 3 h. After incubation, cells were washed twice with 4°C phosphate-buffered saline (PBS; Ca^{2+}/Mg^{2+}-free) and PIP_2 mass was quantitated on a per cell basis, as described [6, 14]. In brief, cells were lysed and lipids were precipitated in trichloroacetic acid and extracted in chloroform. The lipids were then hydrolyzed in KOH. The amount of IP_3 released from the lipid during base hydrolysis was determined using a competitive binding assay (Amersham) and used to calculate the amount of PIP_2 in the original sample. Cell number determinations were carried out in parallel wells using a cellular acid phosphatase assay [15].

Procedures for isolating whole cytoskeleton and focal adhesion complexes. Total cellular protein was isolated from whole cells after culture in suspension or attachment to FN-coated dishes for 30 min by washing the cells twice in PBS (Ca^{2+}/Mg^{2+}-free) at 4°C and then lysing the cells in RIPA buffer (1% Triton X-100, 1% deoxycholate, 0.1% SDS, 150 mM NaCl, 50 mM Tris, pH 7.2) [12, 16]. Whole cytoskeleton samples were obtained from suspended cells by extracting cells in a cytoskeleton-stabilizing buffer (50 mM NaCl, 150 mM sucrose, 3 mM $MgCl_2$, 10 mM Pipes, pH 6.8) containing 0.5% Triton X-100 [16]. Whole cytoskeleton and a subset of this fraction that is highly enriched for known FAC components (e.g., β_1 integrin, talin, vinculin, paxillin, α-actinin, FAK kinase) were isolated from cells bound to magnetic RGD beads using a recently described magnetic isolation technique [12, 16]. In brief, cells were incubated with RGD-coated microbeads (10 per cell) for 30 min at 37°C in a test tube on an inverting platform. The bead-bound cells were then collected and extracted in the Triton-containing cytoskeleton-stabilizing buffer. Aliquots collected at this point in the procedure were defined as "whole cytoskeleton" preparations (i.e., from bead-bound cells). The samples were then subjected to sonication and dounce homogenization. The FAC proteins which remained attached to the beads were isolated from the remainder of the insoluble cytoskeletal components by magnetically pulling the beads to the side wall of the test tube. All samples were solubilized in RIPA buffer and cleared at 13,000 rpm in a microfuge prior to analysis. Protein concentrations were determined using the BCA assay system from Pierce. The samples were diluted with RIPA buffer to obtain identical concentrations for kinase assays. All cell lysing and extraction buffers contained protease inhibitors (2 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin; Boehringer-Mannheim), (100 μM AEBSF; Calbiochem), and 200 μM sodium orthovanadate (Sigma).

Analysis of inositol lipid kinase activity. Lipid kinase assay methods were adapted from Ling *et al.* [17]. Equal protein samples in 5 μl of RIPA buffer were added to a final volume of 50 μl in the assay buffer containing phosphatidyl inositol (PI)/phosphatidylinositol 4-monophosphate (PI4P)/phosphatidylserine (PS) (Sigma) liposomes to provide a 1:10 dilution of detergents. Final concentrations in assay were 30 mM Tris–HCl, pH 7.4, 0.1% Triton X-100, 0.1% deoxycholate, 0.01% SDS, 15 mM NaCl, 1 mM DTT, 1 mM EDTA, 0.1 mM EGTA, 80 μM PI, 80 μM PIP, 80 μM PS, 50 μM ATP, 8 μCi/ml [γ^{32}P]ATP (3000 Ci/mmol), 5 mM $MgCl_2$. Samples were incubated at room temperature for 5 min after the addition of ATP and then the reaction was stopped by addition of 1 ml cold 1 M TCA. Lipids were extracted as in Chilvers *et al.* [14] and analyzed by thin layer chromatography (TLC) [18]. Quantitation of ^{32}P incorporated into inositol lipids that were separated on TLC plates was carried out using a Molecular Dynamics phosphorimager.

RESULTS

C3H 10T1/2 fibroblasts exhibit an increase in PIP_2 synthesis within minutes and a rise in total cellular
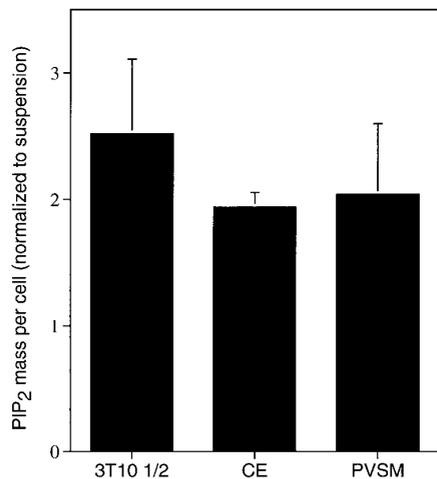
FIG. 1.   C3H10T1/2 fibroblasts (3T10 1/2), capillary endothelial cells (CE), and pulmonary vascular smooth muscle cells (PVSM) all responded to attachment to FN by increasing their total PIP_2 mass in the absence of any exogenous growth factor. Cells were either cultured in suspension or plated on FN-coated dishes for 3 h. Total PIP_2 mass per cell was determined as described under Materials and Methods. All data presented represent mean ± SEM. In all figures, PIP_2 mass per cell is normalized to a value of 1 for the same cells maintained in suspension.

PIP_2 mass within hours when they attach to FN-coated dishes whereas cells maintained in suspension in parallel actually display a net decrease in total PIP_2 mass [6]. To explore whether the major cellular components of the vascular wall exhibit similar adhesion-dependent control of inositol lipid signaling, we measured the effects of ECM binding on total PIP_2 mass in CE cells and VSM cells as well as in the C3H 10T1/2 fibroblasts that we used in our previous study. Measurements were carried out using cells that were cultured on FN-coated dishes in serum-free medium in the complete absence of exogenous growth factors for 3 h. Control cells were held in suspension in the absence of contact with ECM for the same time. Under these conditions, total cellular PIP_2 mass increased approximately two- to threefold in cells attached to FN relative to suspended cells, regardless of whether fibroblasts, CE cells, or VSM cells were utilized (Fig. 1). Thus, FN-dependent control of PIP_2 mass appears to be a signaling mechanism that is common to all three cell types.

To further examine the molecular basis of this ECM-dependent increase in PIP_2, we focused on the mechanism of regulation in CE cells. The effects of ECM binding on multiple integrin signaling pathways have been previously characterized in detail in these cells [16, 19, 20]. We first asked whether the ability of ECM to increase total cellular PIP_2 mass was limited to FN. When CE cells were plated on dishes coated with FN, basement membrane collagen type IV, or interstitial collagen type I, a similar two- to threefold increase PIP_2

mass was observed (Fig. 2). In contrast, only minimal effects on PIP_2 mass were observed when CE cells attached to dishes coated with the nonspecific cell attachment molecule, polylysine. The possibility that integrins mediate this signaling response was supported by the finding that cell binding to dishes coated with a synthetic RGD-containing peptide that possesses only the integrin-binding portion of FN resulted in a similar increase in PIP_2 mass (Fig. 2).

To further investigate the role of integrin receptors in ECM-dependent inositol lipid signaling, PIP_2 levels were measured in CE cells and VSM cells that were cultured in suspension in the presence of either soluble FN (20 μg/ml) or microbeads (4.5 μm diameter) coated with specific integrin ligands, such as RGD peptide or anti-β1 integrin antibody (Fig. 3). Soluble FN promotes integrin occupancy alone whereas microbeads coated with integrin ligands induce both integrin receptor occupancy and clustering [8, 12, 20]. PIP_2 levels did not differ in cells suspended in the presence of soluble FN versus control cells. In contrast, cell binding to microbeads coated with RGD-peptide and anti-β1 integrin antibody increased total PIP_2 mass in CE cells (Fig. 3A) and VSM cells (Fig. 3B) by 60 and 150%, respectively. We currently do not have antibodies that recognize mouse β1 integrin (the CE cells and VSM cells are bovine), however, the mouse fibroblasts exhibited a similar (220%) increase in PIP_2 mass when they were allowed to bind to RGD beads (Fig. 3C). The require-
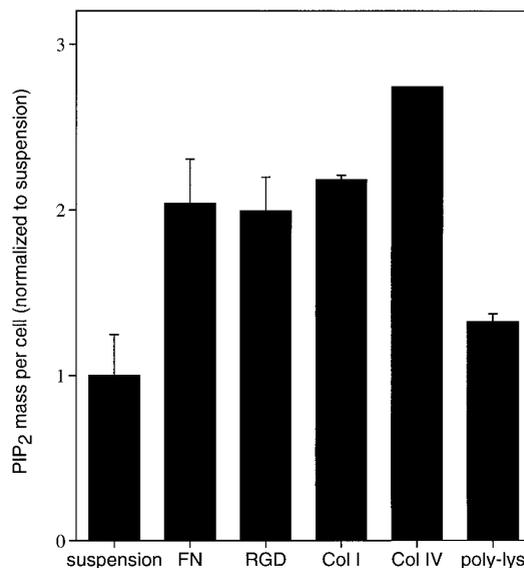

FIG. 2.   Multiple ECM molecules induced an increase in PIP_2 mass in CE cells. CE cells were either cultured in suspension or plated onto dishes coated with FN, RGD peptide (RGD), collagen I (Col I), collagen IV (Col IV), or poly-lysine (poly-lys) for 3 h. Values obtained in cells on FN, RGD, Col I, and Col IV differed significantly ($P \leq 0.05$) from those measured in suspended cells, as determined using an unpaired t test.
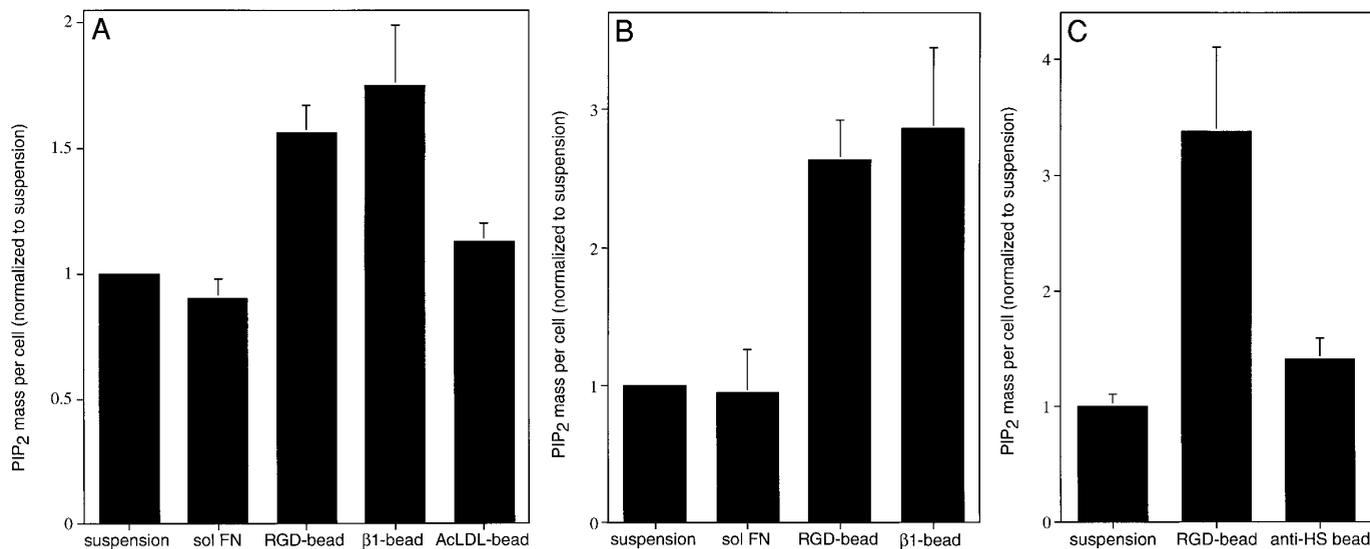
FIG. 3. Integrin receptor clustering and not occupancy alone was required to produce an increase PIP_2 mass. Suspended CE cells (A), VSM cells (B), or C3H 10T1/2 fibroblasts (C) were cultured for 3 h in defined medium containing either no additives (suspension), soluble FN (sol FN), or microbeads coated with synthetic RGD peptide (RGD-bead), AcLDL (AcLDL bead), antibodies against $\beta 1$ integrin ($\beta 1$-bead), or antibodies against heparan sulfate (anti-HS bead). Values measured in cells bound to RGD beads and $\beta 1$ beads differed significantly ($P < 0.05$) from those obtained in suspended cells, as determined using a one tailed t test.

ment for integrins per se in the inositol signaling response was confirmed in CE cells by the finding that cell binding to microbeads coated with AcLDL had no effect on PIP_2 mass (Fig. 3A). AcLDL is a specific ligand for a transmembrane protein that is a metabolic "scavenger" receptor rather than an adhesion receptor. Similarly, beads coated with anti-heparan sulfate antibodies also had little effect on PIP_2 mass in fibroblasts, even though these cells bound avidly to these beads (Fig. 3C).

Our past studies with fibroblasts suggested that attachment to FN may increase PIP_2 mass by stimulating PIP_2 synthesis [6]. In these studies, cells prelabeled with $^{32}PO_4^-$ demonstrated an increase in ^{32}P labeling of cellular PIP_2 upon attachment to FN, which was maximal by 30 min. In order to determine whether attachment to ECM regulates synthesis of PIP_2, we investigated how PIP kinase is regulated in CE cells. This enzyme catalyzes the conversion of phosphatidylinositol monophosphate (PIP) to PIP_2. Based on the studies mentioned above, we assayed PI and PIP kinase activities in CE cells that were either held in suspension or allowed to attach to FN-coated dishes for 30 min. Lipid kinase activities were measured *in vitro* by incubating equal protein samples from cell lysates in the presence of unlabeled PI and PI4P substrates and $[\gamma-^{32}P]ATP$ (Fig. 4). The presence of detergent in the cell lysates should strongly inhibit PI3-kinase activity [21, 22]. The labeled products therefore most likely result from the action of active PI4-kinase and PI4P-5-kinase within the preparations. Interestingly, cell attachment to FN

had no effect on total PIP kinase activity when whole cell lysates were analyzed, indicating that the observed increase in total cellular PIP_2 mass did not result from cell-wide alterations in PIP kinase activity. Furthermore, PI kinase activity in the lysates was also unaffected by adhesion.
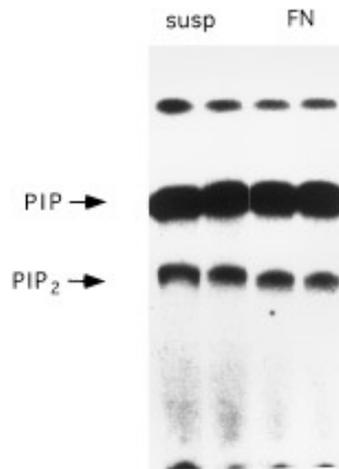

FIG. 4. Binding to FN did not alter total cellular PIP kinase activity in CE cells. Autoradiograph of a thin layer chromatography plate showing ^{32}P-containing lipids that became labelled in the *in vitro* PI/PIP kinase assay. Equal protein samples from cells that were held in suspension (susp) or allowed to bind to FN-coated dishes (FN) for 3 h were assayed *in vitro* for PI and PIP kinase activities. Locations of PI and PIP kinase products (PIP and PIP_2, respectively) are indicated at the left. The top spot which runs above PIP is most likely phosphatidic acid [23].

Previous studies have demonstrated that a subfraction of PIP kinase associates with the insoluble cytoskeleton in other cell types and that this activity increases in response to growth factor stimulation [23, 24]. To investigate whether ECM binding may regulate PIP kinase activity by altering its association with the cytoskeleton, we assayed PI and PIP kinase activities in whole cytoskeleton fractions that were isolated from CE cells cultured in suspension for 30 min in the presence or absence of RGD-coated microbeads (i.e., in the presence or absence of FAC formation). Again, when equal protein samples were analyzed using the *in vitro* inositol lipid kinase assay, no change in total cytoskeletal-associated PIP kinase activity could be detected when cells bound RGD beads (Fig. 5A).

All of the experimental conditions that increased PIP_2 mass in the present study (i.e., cell binding to ECM-coated dishes or to microbeads coated with integrin ligands) have been shown to induce FAC formation [12, 16, 25, 26]. Furthermore, FACs that form in response to CE cell binding to RGD-coated microbeads retain active PIP kinases when analyzed *in vitro* after being physically (magnetically) isolated away from the remainder of the cell and cytoskeleton [16]. Thus, another possibility is that ECM binding may increase total cellular PIP_2 by activating a subset of PIP kinases directly at the site of integrin binding within the FAC. When FAC complexes that formed in response to CE cell binding to RGD beads were magnetically isolated and analyzed *in vitro,* PIP kinase activity was found to be enriched more than twofold in the isolated FACs relative to the whole cytoskeleton isolated from similar bead-bound cells or from control cells cultured in the absence of any ECM ligand (Figs. 5A and 5B). This effect appeared to be specific for PIP kinase since activity of the related enzyme, PI kinase, was not enriched, although it was present in the FAC (not shown). Finally, this mechanism of regulation appeared to be shared by all three cell types since FAC fractions isolated from C3H 10T1/2 fibibroblasts and VSM cells also were enriched for PIP kinase activity when compared on an equal protein basis to their corresponding whole cytoskeleton fractions (not shown).

DISCUSSION

To summarize, these data indicate that inositol lipid turnover, a major chemical signaling pathway that is utilized by soluble growth factors to control cell function, is also controlled by ECM. However, ECM molecules differ from soluble factors in that they utilize transmembrane integrin receptors, rather than growth factor receptors, to exert their effects. Furthermore, while growth factors commonly act by modulating PLC and affecting the degradative part of the turnover loop, ECM and integrins control the availability of the sub-
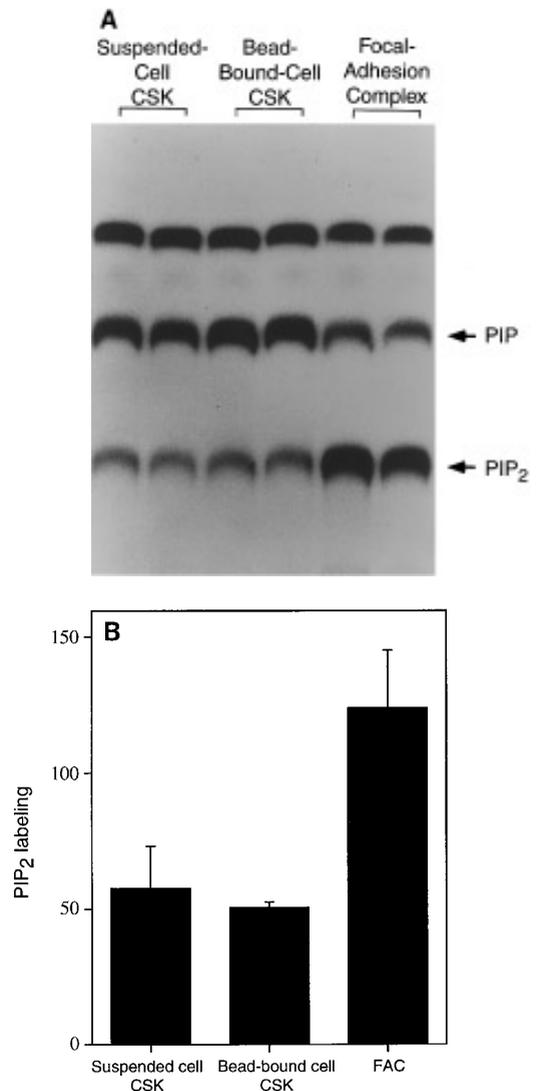

FIG. 5.   PIP kinase activity is enriched in the FAC compared with the whole cytoskeleton fraction isolated from suspended CE cells bound to RGD beads or control cells cultured in the absence of any ECM ligand. (A) Autoradiograph from a representative experiment showing PIP kinase activities present in different subcellular fractions that were analyzed and compared on an equal protein basis using an *in vitro* kinase assay in conjunction with TLC. (B) Densitometric quantitation of the amount of ^{32}P incorporated into PIP_2, as determined by phosphorimage analysis of TLC separation. Suspended-cell CSK, whole cytoskeleton isolated from cells suspended in the absence of any ECM ligand; bead-bound-cell CSK, whole cytoskeleton isolated from suspended cells bound to RGD-beads; focal-adhesion complex, FAC subfraction isolated from the whole cytoskeletal preparation obtained from suspended cells bound to RGD beads.

strate for PLC, PIP_2, by regulating the activity of PIP kinases. Importantly, the net increase in PIP_2 mass was not induced by integrin occupancy alone, rather integrin clustering was required. In addition, our data suggest that this effect may be mediated by a local increase in PIP kinase activity at the site of integrin

binding within the FAC instead of a generalized increase in activation of this enzyme throughout the cell. Thus, this provides a potential explanation for how growth factors and ECM work hand-in-hand to regulate cell metabolism and promote cell growth. Cell adhesion to ECM and integrin clustering apparently prime the cell by inducing synthesis of PIP_2 whereas growth factors drive downstream signaling by triggering its hydrolysis. This may, in part, explain why both types of stimuli are necessary for control of cell growth, viability, and function.

Integrin-dependent regulation of cellular PIP_2 levels and hence, inositol lipid signaling, appears to represent a common mechanism for control of metabolism and function, especially in the cells that comprise the vascular wall. CE cells, VSM cells, and fibroblasts all responded to binding to ECM by similarly increasing PIP_2 mass in the absence of any exogenous growth factors. Furthermore, several ECM molecules that utilize different combinations of integrin α and β subunits to mediate their effects also produced a similar increase in cellular PIP_2. We should note, however, that all of the ECM molecules and antibodies used in this study may interact with cells, in part, through common binding of $\beta1$ integrin receptors. Future studies should address whether clustering of other types of β integrins (i.e., $\beta3$, $\beta5$) have similar effects on these cells. It is also important to note that other cells (e.g., glomerular epithelial cells) have been reported to reduce their PIP_2 levels when they bind to ECM-coated dishes [27]. This may represent a cell type-specific effect of ECM or it could result from simultaneous stimulation of inositol lipid synthesis and degradation by ECM and soluble growth factors, respectively, since those studies were carried out in the presence of serum. Cell binding to ECM also has been reported to stimulate PIP_2 hydrolysis and/or calcium release in other cell types [28–32]. Again, this response may result from simultaneous stimulation by ECM and soluble growth factors or depend on the particular ECM receptor that is ligated.

Other investigators reported that many components of the inositol lipid signaling pathway, including PLC, PI and PIP kinases, and DAG kinase, associate with the cytoskeleton in certain cells [23, 24]. These authors suggested that immobilization of these different components on the cytoskeleton may organize them in close proximity to each other and thus, provide greater efficiency in signal transduction. The results of the present study go further and implicate a specialized region in the cytoskeleton, the FAC, as a site that is enriched for PIP kinase activity and hence, inositol lipid synthesis. However, our data cannot currently distinguish whether the enrichment of activity we measured within isolated FACs is due to local activation PIP kinase or to recruitment and local concentration of active enzyme at the site of integrin binding. A PIP kinase has been

recently cloned [33] and thus, reagents may be available to address this question directly in the near future.

In any case, the position of PIP kinase at the site of ECM binding may place it in close proximity to its signaling partners as well as physiological substrates at the plasma membrane. For example, using the same RGD beads and cytoskeletal fractionation procedures, we have recently shown that in addition to containing active inositol lipid kinases, isolated FACs also are enriched for other integrin-dependent and growth factor-dependent signaling activities (e.g., FAK kinase, c-src) as well as components that mediate inositol lipid breakdown, including PLC and a growth factor receptor (the high-affinity FGF receptor, *flg*) [16]. Other investigators also have localized protein kinase C to FACs within adherent cells [34, 35]. Furthermore, antibodies against PIP_2 stain focal adhesions and detect the FAC proteins α-actinin and vinculin in immunoblots [36]. Taken together, these results suggest that the FAC may serve as a major site of inositol lipid turnover in the cell.

Integrin-mediated effects on PIP_2 mass also may mediate some of the effects of adhesion on the actin cytoskeleton and on cell shape. PIP_2 binds to and regulates the activity of many actin-binding proteins, including gelsolin, profilin, and α-actinin [37]. Based on these effects, an increase in PIP_2 concentration has been predicted to lead to uncapping and increased polymerization of actin filaments [38, 39]. In fact, cultured cells do exhibit a rapid wave of actin polymerization in response to cell binding to ECM-coated dishes [40] that closely parallels the time course of PIP_2 synthesis that is induced by cell binding to ECM [6]. The finding that PIP kinase activity is enriched at the site of integrin binding raises the possibility that production of PIP_2 also may facilitate FAC assembly by promoting actin polymerization locally.

In summary, our data show that integrin-dependent control of inositol lipid synthesis may represent a common mechanism by which ECM modulates cell sensitivity to soluble hormones and regulates cell function, especially in cells of the vascular wall. Because inositol lipids play a key role in signal transduction as well as cytoskeletal organization, understanding of how this pathway is activated may lead to new insights into how cell structure and function are integrated during vessel development.

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