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Rapid Communication

Differential Regulation of Focal Adhesion Kinase and Mitogen-Activated Protein Kinase Tyrosine Phosphorylation During Insulin-Like Growth Factor-I-Mediated Cytoskeletal Reorganization

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Abstract: In SH-SY5Y human neuroblastoma cells, insulin-like growth factor (IGF)-I mediates membrane ruffling and growth cone extension. We have previously shown that IGF-I activates the tyrosine phosphorylation of focal adhesion kinase (FAK) and extracellular signal-regulated protein kinase (ERK) 2. In the current study, we examined which signaling pathway underlies IGF-I-mediated FAK phosphorylation and cytoskeletal changes and determined if an intact cytoskeleton was required for IGF-I signaling. Treatment of SH-SY5Y cells with cytochalasin D disrupted the actin cytoskeleton and prevented any morphological changes induced by IGF-I. Inhibitors of phosphatidylinositol 3-kinase (PI 3-K) blocked IGF-I-mediated changes in the actin cytoskeleton as measured by membrane ruffling. In contrast, PD98059, a selective inhibitor of ERK kinase, had no effect on IGF-I-induced membrane ruffling. In parallel with effects on the actin cytoskeleton, cytochalasin D and PI 3-K inhibitors blocked IGF-I-induced FAK tyrosine phosphorylation, whereas PD98059 had no effect. It is interesting that cytochalasin D did not block IGF-I-induced ERK2 tyrosine phosphorylation. Therefore, it is likely that FAK and ERK2 tyrosine phosphorylations are regulated by separate pathways during IGF-I signaling. Our study suggests that integrity as well as dynamic motility of the actin cytoskeleton mediated by PI 3-K is required for IGF-I-induced FAK tyrosine phosphorylation, but not for ERK2 activation. **Key Words:** Insulin-like growth factor I—Actin cytoskeleton—Focal adhesion kinase—Extracellular signal-regulated protein kinase—Phosphatidylinositol 3-kinase.
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Insulin-like growth factor (IGF)-I is a polypeptide that plays an essential role in embryonic and early postnatal development (Zackenfels et al., 1995). In the nervous system, IGF-I is important for cytoskeletal organization and extension of growth cones. These changes in neuronal architecture require IGF-I-mediated redistribution of the actin cytoskeleton (Kadowaki et al., 1986; Leventhal et al., 1997).

We are interested in the downstream signaling pathways activated by IGF-I during nervous system growth. We have reported that IGF-I binds to the type I IGF receptor and activates the mitogen-activated protein (MAP) kinase and phosphatidylinositol 3-kinase (PI 3-K) pathways (Kim et al., 1997; Leventhal and Feldman, 1997; Leventhal et al., 1997; Cheng and Feldman, 1998). Our work suggests that the physiological effects of each pathway are separable. For example, IGF-I activation of extracellular signal-regulated protein kinase (ERK) 2, the most studied member of the MAP kinase family, results in neuronal differentiation (Kim et al., 1997), whereas IGF-I blocks neuronal apoptosis via PI 3-K signaling (Singleton et al., 1996). We do not know the relationship between these two pathways and the ability of IGF-I to reorganize the actin cytoskeleton. Furthermore, it is not known if cytoskeletal organization is a prerequisite for one or more aspects of IGF-I-mediated signaling.

To answer these questions, we initially investigated one downstream target of PI 3-K, focal adhesion kinase (FAK). FAK is a cytoplasmic tyrosine kinase (Ilic et al., 1997; Zachary, 1997) instrumental in growth factor-mediated changes in cytoskeletal organization (reviewed by Carpenter and Cantley, 1996). IGF-I and insulin can either phosphorylate or dephosphorylate FAK, depending on the cell type and the adhesion status (Pillay et al., 1995; Leventhal et al., 1997; Baron et al., 1998). We have found that IGF-I stimulates the tyrosine phosphorylation of FAK in neurons as growth cones advance over the substrate (Leventhal et al., 1997).

In the current study, we examined which signaling pathway underlies IGF-I-mediated FAK phosphorylation and cytoskeletal changes and determined if an intact cytoskeleton was required for IGF-I signaling. In our experiments, we used SH-SY5Y human neuroblastoma cells, a well-studied in vitro model of neuronal growth and differentiation (Feldman and Randolph, 1991; Lavenius et al., 1994; Leventhal et al., 1995). We report that IGF-I-mediated FAK tyrosine phosphorylation and changes in neuronal morphology require PI 3-K signaling. We also found that disruption of the actin cytoskeleton blocks IGF-I activation of FAK but has no effect on IGF-I activation of MAP kinase pathways. These results are discussed in terms of a general model of growth factor-mediated changes in neuronal architecture.

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Abbreviations used: ECM, extracellular matrix; ERK, extracellular signal-regulated protein kinase; FAK, focal adhesion kinase; IGF, insulin-like growth factor; MAP, mitogen-activated protein; PI 3-K, phosphatidylinositol 3-kinase.

MATERIALS AND METHODS

Materials

Anti-phosphotyrosine antibodies were purchased as follows: PY20 from Transduction Laboratories (Lexington, KY, U.S.A.) and 4G10 from Upstate Biochemicals (Lake Placid, NY, U.S.A.). Anti-FAK and anti-ERK2 polyclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). LY294002 and wortmannin were purchased from Biomol (Plymouth Meeting, PA, U.S.A.). Cytochalasin D was purchased from Calbiochem (San Diego, CA, U.S.A.). PD98059 was kindly provided by Dr. A. Saltiel of Parke-Davis Pharmaceutical Research (Ann Arbor, MI, U.S.A.). Other reagents were purchased from Sigma (St. Louis, MO, U.S.A.) or Boehringer-Mannheim (Indianapolis, IN, U.S.A.).

Cell culture

SH-SY5Y human neuroblastoma cells were grown in Dulbecco's modified Eagle's medium containing 10% calf serum and maintained at 37°C in a humidified atmosphere with 10% CO_2. For immunocytochemistry experiments, cells were grown on glass coverslips. At 18–24 h before experiments, medium was replaced with Dulbecco's modified Eagle's medium without serum.

Immunoprecipitation, immunoblotting, and immunocytochemistry

Immunoprecipitation and immunoblotting were performed as described previously (Kim et al., 1997). All experiments were repeated at least twice, and typical representative results are shown in the figures. Immunocytochemistry was performed as described previously (Leventhal et al., 1997). SH-SY5Y cells grown on glass coverslips were fixed in 4% paraformaldehyde and permeabilized in 0.15% Triton X-100 plus 1% bovine serum albumin in phosphate-buffered saline, pH 7.2. Actin filaments were stained for 15 min with 2 units/ml rhodamine–phalloidin (Molecular Probes, Eugene, OR, U.S.A.).

RESULTS

We first examined the organization of the actin filaments in SH-SY5Y cells by staining with rhodamine-labeled phalloidin. In control cells, we could detect a distinct organization of actin cytoskeleton, including stress fibers and filopodia (Fig. 1A). Actin filaments were especially concentrated at the periphery of the cells. In agreement with our previous results (Leventhal et al., 1997), treatment of IGF-I resulted in the extension of growth cones. When these cells were stained with rhodamine–phalloidin, there was a dense staining of actin filaments at the leading membrane edges, representing membrane ruffling (Fig. 1B). Cytochalasin D treatment produced a gross disruption of the actin cytoskeleton with rounding up of the cells (Fig. 1C). Actin filaments were redistributed into discrete round patches scattered randomly over the cells. IGF-I treatment had no effect on these changes. Treatment with two PI 3-K inhibitors alone, LY294002 and wortmannin, had little effect on the actin cytoskeleton; we did not observe differences in actin stress fibers of cells treated with wortmannin versus LY294002 (data not shown). Stress fibers and filopodia were also maintained. However, these compounds prevented growth cone extension and membrane ruffling after IGF-I treatment (Fig. 1D and E). In contrast, PD98059, an inhibitor of MAP kinase kinase, did not block IGF-I-induced growth cone extension and membrane ruffling (Fig. 1F) at doses up to 100 μM (data not shown).

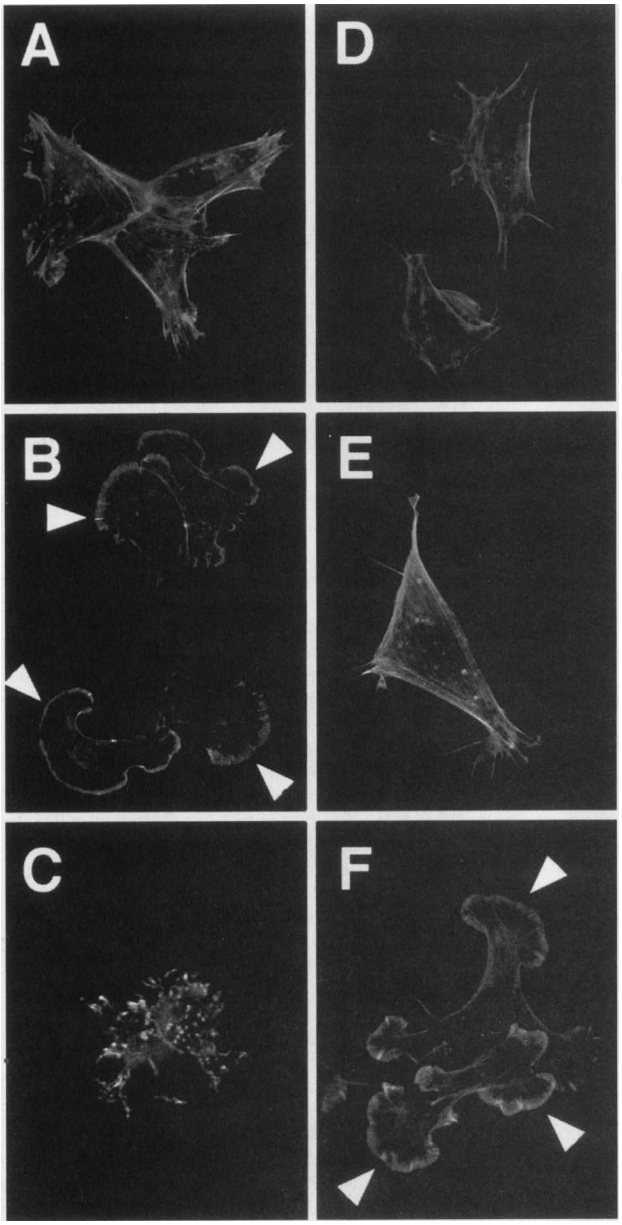

FIG. 1. Effect of cytochalasin D and inhibitors of PI 3-K and MAP kinase on IGF-I-mediated membrane ruffling. Serum-starved SH-SY5Y cells were treated without (**A** and **B**) or with 1 mM cytochalasin D (**C**), 10 μM LY294002 (**D**), 100 nM wortmannin (**E**), or 10 μM PD98059 (**F**) before a 5-min incubation with 10 nM IGF-I (B–F). Cells were fixed and stained with rhodamine–phalloidin to visualize actin filaments. Arrowheads indicate the membrane ruffling.

Next we examined the changes in tyrosine phosphorylation of FAK and ERK2. In agreement with our previous reports (Kim et al., 1997; Leventhal et al., 1997), IGF-I treatment resulted in increased tyrosine phosphorylation of FAK and ERK2 (Fig. 2). Treatment of cytochalasin D completely abolished IGF-I-induced FAK tyrosine phosphorylation (Fig. 2A). In contrast, cytochalasin D had no effect on ERK2 tyrosine

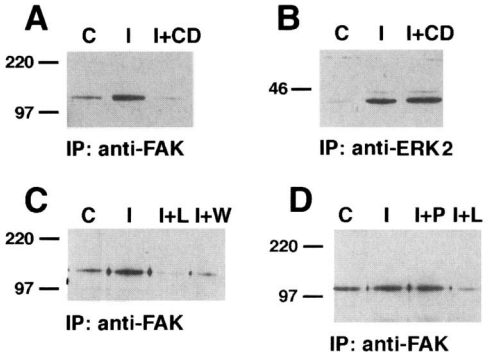

FIG. 2. Effect of cytochalasin D and inhibitors of PI 3-K and MAP kinase on IGF-I-induced tyrosine phosphorylation of FAK and ERK2. Cells were treated as in Fig. 1 and incubated with 10 n*M* IGF-I for 30 min. Cell lysates were immunoprecipitated with antibodies against FAK (**A, C,** and **D**) or ERK2 (**B**) and then immunoblotted with anti-phosphotyrosine antibodies. C, control; I, IGF-I; CD, cytochalasin D; L, LY294002; W, wortmannin; P, PD98059; IP, immunoprecipitation.

phosphorylation (Fig. 2B). Recent reports show that PI 3-K activity is required for platelet-derived growth factor-induced FAK tyrosine phosphorylation (Rankin et al., 1996; Saito et al., 1996). In agreement with these reports, IGF-I-induced FAK tyrosine phosphorylation was blocked by treatment of the cells with the PI 3-K inhibitors LY294002 and wortmannin (Fig. 2C). In contrast, PD98059, which inhibits IGF-I-induced ERK2 activity (Kim et al., 1997), had no effect on FAK tyrosine phosphorylation (Fig. 2D).

DISCUSSION

In the current study, we investigated which downstream signaling pathway mediates the ability of IGF-I to reorganize the actin cytoskeleton and produce membrane ruffling in neurons. We found that two structurally unrelated PI 3-K inhibitors, LY294002 and wortmannin, blocked IGF-I-mediated membrane ruffling. In contrast, PD98059, which can effectively block IGF-I activation of ERK2 (Kim et al., 1997), had no effect on the ability of IGF-I to produce ruffled membranes. These data indicate that IGF-I-mediated changes in actin reorganization and the neuronal cytoskeleton are mediated via PI 3-K signaling. Our results agree with reports in nonneuronal cells. In fibroblasts, membrane ruffling is stimulated by several growth factors, including IGF-I (Kotani et al., 1994; Wennström et al., 1994). In nonneuronal cells, activation of PI 3-K is necessary for membrane ruffling by acting as an upstream regulator of the small GTPase protein, Rac (Kotani et al., 1994; Nishiyama et al., 1994; Wennström et al., 1994; Hawkins et al., 1995; Parker, 1995). We are currently investigating the role of PI 3-K activation of Rac in IGF-I-mediated changes in neuronal architecture (Kim et al., 1996).

In this report, we also show that tyrosine phosphorylation of FAK and ERK2 is differentially regulated by cytochalasin D. IGF-I activation of FAK is blocked by cytochalasin D, whereas the same treatment has no effect on IGF-I-mediated ERK2 activation. FAK can be activated by integrin clustering, adhesion to the extracellular matrix (ECM), and growth factor stimulation (Ilic et al., 1997; Zachary, 1997). We have also reported that binding of SH-SY5Y cells with laminin can induce FAK tyrosine phosphorylation (Leventhal and Feldman, 1996*b*; Leventhal et al., 1997). Our current study suggests that integrity of actin filaments is critical for FAK, but not ERK2, activation in neurons and that FAK and ERK2 tyrosine phosphorylations are regulated by separate pathways during IGF-I stimulation. These data imply that first an external stimulus, provided by a growth factor, integrin–ECM binding, or both, reorganizes the actin cytoskeleton. Cytoskeletal rearrangement activates proteins like FAK, which form focal adhesions between the cell and ECM (Leventhal and Feldman, 1997). In contrast, ERK2 is directly activated by IGF-I and does not require changes in neuronal architecture for IGF-I-mediated activity.

Our results point out interesting differences between nonneuronal and neuronal cells. In fibroblasts, IGF-I *deactivates* FAK as cells attach and organize their cytoskeleton (Baron et al., 1998). In contrast, we find that IGF-I *activates* FAK as neurons attach and organize their cytoskeleton. Our data emphasize the importance of an intact cytoskeleton for growth factor-mediated FAK activation in neurons. Unlike fibroblasts, neurons must form growth cones, which protrude into the substratum and form the pathway for neurites. Our results suggest that activation of FAK promotes the formation of focal adhesions in the organized neuronal actin cytoskeleton, permitting attachment and extension of growth cones required for neurite outgrowth. Thus, in neurons, FAK provides a unique point of convergence among organization of the actin cytoskeleton, growth factor signaling, and formation of focal adhesions.

The current study does not address the question of whether or not integrin–ligand binding activates MAP kinases in SH-SY5Y cells. However, our previous studies suggest integrin–ECM binding does not activate this pathway (Leventhal and Feldman, 1996*a,b*). In the current study, we demonstrate that cytoskeletal disruption has no effect on growth factor-mediated MAP kinase activation. These new results again emphasize the differences between nonneuronal cells and neurons. In contrast to our data in neurons, reports in fibroblasts suggest integrin binding with ECM activates MAP kinases (Chen et al., 1994; Morino et al., 1995) and overexpression of FAK enhances fibronectin-stimulated ERK2 activation (Schlaepfer and Hunter, 1997). Recently, however two studies suggest the existence of independent integrin signaling pathways in fibroblasts, one of which can activate FAK and one of which affects MAP kinase (Seufferlein et al., 1996; Lin et al., 1997). These ideas agree more with our own observations that MAP kinase and FAK tyrosine phosphorylations are regulated by separate pathways.

In the current study, the PI 3-K inhibitor LY294002 blocked IGF-I-mediated membrane ruffling. We also found that LY294002 and wortmannin block IGF-I-induced FAK tyrosine phosphorylation. In contrast, PD98059 had no effect. These results are in agreement with recent reports where FAK associates with PI 3-K and PI 3-K activity is required for platelet-derived growth factor-induced FAK tyrosine phosphorylation (Chen and Guan, 1994*a,b*; Rankin et al., 1996; Saito et al., 1996). Collectively, our data and those of others (Chen and Guan, 1994*a,b*; Rankin et al., 1996; Saito et al., 1996) suggest that not only integrity but also dynamic motility of the actin cytoskeleton is required for IGF-I-induced FAK tyrosine phosphorylation.

Our findings in neurons are also important in the context of recent reports, including our own (Singleton et al., 1996), that growth factor protection against neuronal apoptosis is mediated via PI 3-K pathways. Accumulating data show a

strong association among apoptosis, cellular adhesion, and the cytoskeleton. FAK is also now known to be a substrate for caspase-3, a protease that serves as a cellular death effector (Widmann et al., 1998). Our results in neurons that both IGF-I-mediated FAK activation and IGF-I protection against apoptosis require PI 3-K activation suggest an interesting link between FAK activation and cellular protection.

In summary, IGF-I mediates changes in neuronal morphology and activation of FAK via PI 3-K signaling. In contrast, MAP kinase signaling is not required for either of these IGF-I-mediated effects. These results suggest the dissociation of IGF-I signaling for FAK and ERK2 tyrosine phosphorylation and in mediating essential changes in neuronal architecture.

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REFERENCES

Baron V., Calléja V., Ferrari P., Alengrin F., and Van Obberghen E. (1998) $p125^{Fak}$ focal adhesion kinase is a substrate for the insulin and insulin-like growth factor-I tyrosine kinase receptors. *J. Biol. Chem.* **273,** 7162–7168.

Carpenter C. L. and Cantley L. C. (1996) Phosphoinositide 3-kinase and the regulation of cell growth. *Biochim. Biophys. Acta* **1288,** M11–M16.

Chen H.-C. and Guan J.-L. (1994*a*) Association of focal adhesion kinase with its potential substrate phosphatidylinositol 3-kinase. *Proc. Natl. Acad. Sci. USA* **91,** 10148–10152.

Chen H.-C. and Guan J.-L. (1994*b*) Stimulation of phosphatidylinositol 3′-kinase association with focal adhesion kinase by platelet-derived growth factor. *J. Biol. Chem.* **269,** 31229–31233.

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.

Cheng H.-L. and Feldman E. L. (1998) Bi-directional regulation of p38 kinase and c-jun N-terminal protein kinase by insulin-like growth factor-I. *J. Biol. Chem.* **273,** 14560–14565.

Feldman E. L. and Randolph A. E. (1991) Mannose 6-phosphate potentiates insulin-like growth factor II effects in cultured human neuroblastoma cells. *Brain Res.* **562,** 111–116.

Hawkins P. T., Eguinoa A., Qui R. G., Stokoe D., Cooke F. T., Wakter R., Wennström S., Claesson W. L., Evans T., Symons M., and Stephens L. (1995) PDGF stimulates an increase in GTP-Rac via the activation of phosphoinositide 3-kinase. *Curr. Biol.* **5,** 393–403.

Ilic D., Damsky C. H., and Yamamoto T. (1997) Focal adhesion kinase: at the crossroads of signal transduction. *J. Cell Sci.* **110,** 401–407.

Kadowaki T., Koyasu S., Nishida E., Sakai H., Takaku F., Yahara I., and Kasuga M. (1986) Insulin-like growth factors, insulin, and epidermal growth factor cause rapid cytoskeletal reorganization in KB cells. *J. Biol. Chem.* **261,** 16141–16147.

Kim B., Leventhal P. S., and Feldman E. L. (1996) Insulin-like growth factor-I (IGF-I) induces localization of RAC and phosphatidylinositol 3-kinase (PI3-K) to membrane ruffles. (Abstr.) *Mol. Biol. Cell* **7,** 342a.

Kim B., Leventhal P. S., Saltiel A. R., and Feldman E. L. (1997) Insulin-like growth factor-I-mediated neurite outgrowth in vitro requires MAP kinase activation. *J. Biol. Chem.* **272,** 21268–21273.

Kotani K., Yonezawa K., Hara K., Ueda H., Kitamura Y., Sakaue H., Ando A., Chavanieu A., Calas B., Grigorescu F., Nishiyama M., Waterfield M. D., and Kasuga M. (1994) Involvement of phosphoinositide 3-kinase in insulin- or IGF-1-induced membrane ruffling. *EMBO J.* **13,** 2313–2321.

Lavenius E., Parrow V., Nånberg E., and Påhlman S. (1994) Basic FGF and IGF-I promote differentiation of human SH-SY5Y neuroblastoma cells in culture. *Growth Factors* **10,** 29–39.

Leventhal P. S. and Feldman E. L. (1996*a*) The tyrosine kinase inhibitor methyl 2,5-dihydroxycinnimate disrupts changes in the actin cytoskeleton required for neurite formation. *Brain Res. Mol. Brain Res.* **43,** 338–340.

Leventhal P. S. and Feldman E. L. (1996*b*) Tyrosine phosphorylation and enhanced expression of paxillin during neuronal differentiation *in vitro*. *J. Biol. Chem.* **271,** 5957–5960.

Leventhal P. S. and Feldman E. L. (1997) Insulin-like growth factors as regulators of cell motility: signaling mechanisms. *Trends Endocrinol. Metab.* **8,** 1–6.

Leventhal P. S., Randolph A. E., Vesbit T. E., Schenone A., Windebank A. J., and Feldman E. L. (1995) Insulin-like growth factor-II as a paracrine growth factor in human neuroblastoma cells. *Exp. Cell Res.* **221,** 179–186.

Leventhal P. S., Shelden E. A., Kim B., and Feldman E. L. (1997) Tyrosine phosphorylation of paxillin and focal adhesion kinase during insulin-like growth factor-I-stimulated lamellipodial advance. *J. Biol. Chem.* **272,** 5214–5218.

Lin T. H., Aplin A. E., Shen Y., Chen Q., Schaller M., Romer L., Aukhil I., and Juliano R. L. (1997) Integrin-mediated activation of MAP kinase is independent of FAK: evidence for dual integrin signaling pathways in fibroblasts. *J. Cell Biol.* **136,** 1385–1395.

Morino N., Mimura T., Hamasaki K., Tobe K., Ueki K., Kikuchi K., Takehara K., Kadowaki T., Yazaki Y., and Nojima Y. (1995) Matrix/integrin interaction activates the mitogen-activated protein kinase, $p44^{erk-1}$ and $p42^{erk-2}$. *J. Biol. Chem.* **270,** 269–273.

Nishiyama T., Sasaki T., Takaishi K., Kato M., Yaku H., Araki K., Matsuura Y., and Takai Y. (1994) *rac* p21 is involved in insulin-induced membrane ruffling and *rho* p21 is involved in hepatocyte growth factor- and 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced membrane ruffling in KB cells. *Mol. Cell. Biol.* **14,** 2447–2456.

Parker P. J. (1995) PI 3-kinase puts GTP on the Rac. *Curr. Biol.* **5,** 577–579.

Pillay T. S., Sasaoka T., and Olefsky J. M. (1995) Insulin stimulates the tyrosine dephosphorylation of pp125 focal adhesion kinase. *J. Biol. Chem.* **270,** 991–994.

Rankin S., Hooshmand-Rad R., Claesson-Welsh L., and Rozengurt E. (1996) Requirement for phosphatidylinositol 3′-kinase activity in platelet-derived growth factor-stimulated tyrosine phosphorylation of p125 focal adhesion kinase and paxillin. *J. Biol. Chem.* **271,** 7829–7834.

Saito Y., Mori S., Yokote K., Kanzaki T., and Morisaki N. (1996) Phosphatidylinositol 3-kinase activity is required for the activation process of focal adhesion kinase by platelet-derived growth factor. *Biochem. Biophys. Res. Commun.* **224,** 23–26.

Schlaepfer D. D. and Hunter T. (1997) Focal adhesion kinase overexpression enhances Ras-dependent integrin signaling to ERK2/mitogen-activated protein kinase through interactions with and activation of c-Src. *J. Biol. Chem.* **272,** 13189–13195.

Seufferlein T., Withers D. J., Mann D., and Rozengurt E. (1996) Dissociation of mitogen-activated protein kinase activation from p125 focal adhesion kinase tyrosine phosphorylation in Swiss 3T3 cells stimulated by bombesin, lysophosphatidic acid, and platelet-derived growth factor. *Mol. Biol. Cell* **7,** 1865–1875.

Singleton J. R., Dixit V. M., and Feldman E. L. (1996) Type I insulin-like growth factor receptor activation regulates apoptotic proteins. *J. Biol. Chem.* **271,** 31791–31794.

Wennström S., Hawkins P., Cooke F., Hara K., Yonezawa K., Kasuga M., Jackson T., Claesson-Welsh L., and Stephens L. (1994) Activation of phosphoinositide 3-kinase is required for PDGF-stimulated membrane ruffling. *Curr. Biol.* **4,** 385–393.

Widmann C., Gibson S., and Johnson G. L. (1998) Caspase-dependent cleavage of signaling proteins during apoptosis. *J. Biol. Chem.* **273,** 7141–7147.

Zachary I. (1997) Focal adhesion kinase. *Int. J. Biochem. Cell Biol.* **29,** 929–934.

Zackenfels K., Oppenheim R. W., and Rohrer H. (1995) Evidence for an important role of IGF-I and IGF-II for the early development of chick sympathetic neurons. *Neuron* **14,** 731–741.