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Anchorage-Independent Activation of Mitogen-Activated Protein Kinase through Phosphatidylinositol-3 Kinase by Insulin-like Growth Factor I

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Insulin-like growth factor I (IGF-I) is a well-established mitogen in human breast cancer cells. We show here that human breast cancer MCF-7 cells, which were prevented from attaching to the substratum and were floating in medium, responded to IGF-I and initiated DNA synthesis. The addition of IGF-I to floating cells induced activation of protein kinase B (PKB)/Akt, as to cells attached to the substratum. In addition, mitogen-activated protein kinase (MAPK)/extracellular response kinase (ERK) and its upstream kinases, ERK kinase (MEK) and Raf-1, were activated by IGF-I in floating cells. While the IGF-I-induced activation of PKB/Akt was inhibited by PI3-K inhibitor LY294002 but not by MEK inhibitor PD98059, the activation of both MEK and ERK by IGF-I was inhibited by both. These findings suggest that the IGF-I signal that leads to stimulation of DNA synthesis of MCF-7 cells is transduced to ERK through PI3-K, only when they are anchorage-deficient.

Anchorage-independent growth is one of the most prominent characteristics of malignantly transformed cells such as metastatic tumor cells or cancerous cells in culture. Nonmalignant cells generally have a property of anchorage-dependent growth and fail to proliferate when they are prevented from attaching on a solid substratum (1, 2). The adhesive interactions between cells and the substratum are mediated by the integrin family of cell surface receptors (3, 4). Integrin-mediated cell adhesion to the substratum produces a great many signals that affect the growth regulatory pathway. These signals induced the activation of tyrosine kinases such as focal adhesion kinase (FAK) (5, 6) and pp60^{src} (7); serine-threonine kinases such as mitogen-activated protein kinase (MAPK)/extracellular response kinase (ERK) (8, 9) and protein kinase C (PKC) (10, 11); the small GTPase Rho (12, 13) and lipid mediators (14, 15). Thus, the integrin-dependent signal transducers are well established as participants involved in growth signaling. In malignantly transformed cells, anchorage independence could be achieved as a consequence of an anchorage-independent activation of a step involved in integrin-mediated adhesion signaling. A number of oncogenes have been found to fit this description.

Insulin-like growth factor I (IGF-I) is a well established mitogen to human breast cancer cells such as MCF-7 cells (16–18). Growth stimulatory signal of IGF-I in MCF-7 cells is transduced through phosphatidylinositol 3-kinase (PI3-K) to protein kinase B (PKB)/Akt when cells are adherent to the culture substratum (19). The soft agar growth assay revealed that colony forming ability of MCF-7 cells is significantly promoted in the presence of serum compared to that of nonmalignant breast epithelial cells (20). This indicates that MCF-7 cells have the ability to achieve anchorage-independent and serum-dependent growth. In the present study, we examined whether anchorage-deficient MCF-7 cells respond to IGF-I or not and how the IGF-I signal is transduced in cells.

MATERIALS AND METHODS

Cell culture. Human breast cancer MCF-7 cells were maintained in RPMI 1640 medium supplemented with 5% FBS (21). For DNA synthesis assay, cells were serum deprived (0.1% serum) for 48 h, and then seeded on plastic dishes or dishes coated with poly-HEME (Sigma) (2). Cells were added with 100 ng/ml of IGF-I (Sigma) and ^{3}H-thymidine at 1 h after seeding, and DNA synthesis was determined 24 h later by measurement of the radioactivity of ^{3}H-thymidine incorporated into the 10% TCA-insoluble materials. In some experiments, LY294002, PD98059, or rapamycin (Calbiochem) was preincubated with cells for 30 min prior to adding IGF-I.

Western blot analysis. For PKB/Akt assay, cells were lysed in 1% SDS in 20 mM Tris–HCl, pH 7.4, 1 mM $CaCl_2$, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 0.2 mM sodium vanadate. Total cell lysates were electrophoresed in 10% acrylamide gels, and Western blotting was performed using anti-phospho-PKB/Akt antibody (New

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England BioLabs), horseradish peroxidase-conjugated secondary antibody, and an enhanced chemiluminescence kit (Amersham Pharmacia). Endogeneous phosphorylation of ERK1/2 was determined by mobility shift of p44/p42 ERKs to their phosphorylated form pp44/pp42. ERK1/2 was immunoprecipitated with anti-ERK antibody (Upstate Biotechnology), electrophoresed on 10% acrylamide gels, and Western blotting was performed using anti-phospho-ERK1/2 antibody (New England BioLabs).

In vitro kinase assays. *In vitro* kinase assays for ERK activation were performed on immunoprecipitated ERK1/2 with ^{32}P-ATP, using myelin basic protein (MBP) (Sigma) as substrate. Phosphorylated MBP was electrophoresed on 15% acrylamide gels, and autoradiography was performed. *In vitro* kinase assays for ERK kinase (MEK) were performed on immunoprecipitated MEK1/2 proteins with anti-MEK antibody (Santa Cruz Biotechnology) using ERK2 (New England BioLabs) as substrate (22). Immunoprecipitated MEK1/2 was incubated with ERK2 and ^{32}P-ATP for 30 min at 30°C. Phosphorylated ERK2 was electrophoresed, and autoradiography was performed. *In vitro* kinase assays for Raf-1 were performed on immunoprecipitated Raf-1 proteins using GST-MEK (Upstate Biotechnology) as substrate (22). Raf-1 was immunnoprecipitated with anti-Raf-1 antibody (Santa Cruz Biotechnology), then incubated with GST-MEK and ^{32}P-ATP for 30 min at 30°C. Phosphorylated GST-MEK was electrophoresed and autoradiographed.

RESULTS

Stimulation of DNA synthesis by IGF-I in floating cells. To determine whether IGF-I stimulates floating MCF-7 cells to initiate DNA synthesis, IGF-I with ^{3}H-thymidine was added to the cells 1 h after seeding on plastic dishes coated with poly-HEME, which prevents cells from attaching to, and spreading on, the substratum (2). After incubating cells for 24 h, the thymidine incorporation of floating cells that were stimulated with 100 ng/ml of IGF-I was 1.7-fold above that of unstimulated cells (Fig. 1A). The addition of 100 ng/ml of IGF-I to cells attached to the substratum 1 h after seeding, followed by a 24 h-incubation, resulted in a 3.6-fold increase in ^{3}H-thymidine incorporation compared to that in unstimulated cells (Fig. 1A). The DNA synthesis-stimulatory effect of IGF-I on floating cells was smaller than that on attached cells, however, the difference in the ^{3}H-thymidine incorporation between stimulated and unstimulated floating cells was significant (Student's t test, $P < 0.01$). During incubation until the onset of DNA synthesis, the cell viability in both cultures with or without IGF-I decreased equally and did not differ significantly 5, 9, and 24 h after seeding (Fig. 1B).

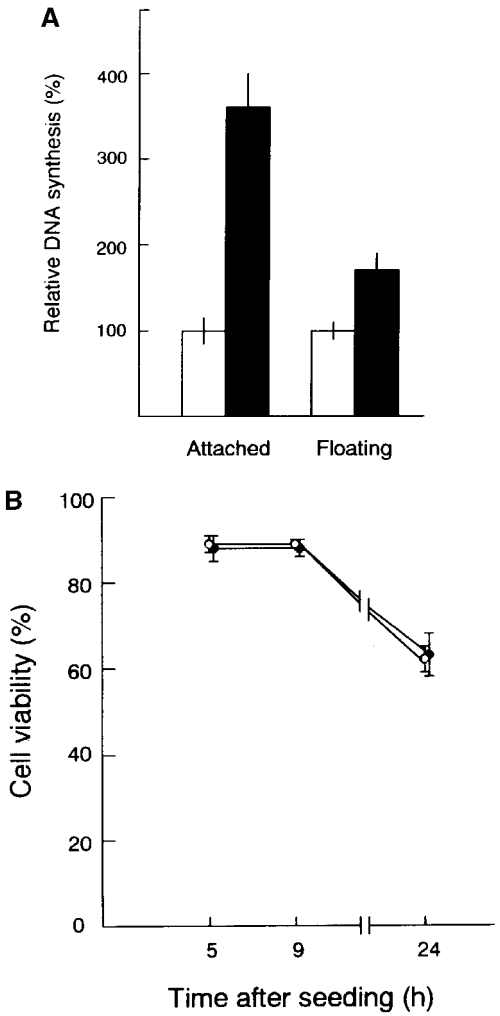

FIG. 1. Enhancement of DNA synthesis by IGF-I in attached and floating MCF-7 cells. (A) At 1 h after seeding on plastic dishes or those coated with poly-HEME, cells were added with (closed columns) or without (open columns) 100 ng/ml of IGF-I and incubated with ^{3}H-thymidine for over 24 h. The mean ± SD of ^{3}H-thymidine incorporation in cells stimulated with IGF-I relative to unstimulated cells of triplicate cultures is shown. (B) Cell viability of floating cells during incubation until the onset of DNA synthesis. The viability of floating cells incubated with (closed circles) or without (open circles) 100 ng/ml of IGF-I was determined at 5, 9, and 24 h after seeding by a dye exclusion test and the mean value ± SD of triplicate cultures is shown.

Inhibition of IGF-I-induced DNA synthesis by enzyme inhibitors. It is known that the IGF-I or insulin signal is transduced from PI3-K to PKB/Akt (19, 24–26) or p70^{S6K} (27), as a downstream signaling molecule. To clarify how the IGF-I signal that leads to stimulation of DNA synthesis in floating cells is transduced, we examined the effects of PI3-K inhibitor LY294002 (28), MEK inhibitor PD98059 (29), or p70^{S6K} inhibitor rapamycin (30), on IGF-I-induced DNA synthesis. Cells at 1 h after seeding were incubated with different concentrations of the inhibitors for 30 min, then incubated with 100 ng/ml of IGF-I with ^{3}H-thymidine for a further 24 h. The concentration of LY294002, PD98059, or rapamycin required for 50% inhibition (IC_{50}) of the IGF-I-induced DNA synthesis in attached cells, estimated from the dose–response curve, was 4 μM, more than 100 μM, and more than 1 nM, respectively (Table 1). Among the inhibitors tested, the IC_{50} of LY294002 for DNA synthesis in attached cells was closest to that for PI3-K (1.4 μM) (28). The IC_{50} of PD98059 or rapamycin for the IGF-I-induced DNA synthesis in attached cells was more than 50-fold or

TABLE 1

Inhibition of IGF-I-induced DNA Synthesis by Enzyme Inhibitors in Attached and Floating MCF-7 Cells

Inhibitor	IC_{50} for DNA synthesis[a]	
	Attached cells	Floating cells
LY294002 (μM)	4	18
PD98059 (μM)	100<	12
Rapamycin (nM)	1<	1<

[a] Cells arrested by culture in 0.1% serum-containing medium for 2 days were harvested and incubated on plastic dishes, or dishes coated with poly-HEME for 1 h. Cells were incubated with different concentrations of inhibitor for 30 min, and then added with 100 ng/ml of IGF-I and ^{3}H-thymidine. After incubation for 24 h, radioactivity incorporated into the acid-insoluble materials was determined. IC_{50} for DNA synthesis was estimated from respective dose-response curve.

20-fold above the IC_{50} for MEK (2 μM) (29) or $p70^{S6K}$(0.05 nM) (30), respectively. In contrast, the IC_{50} of LY294002, PD98059, and rapamycin for the enhancement of DNA synthesis by IGF-I in floating cells was 18 μM, 12 μM, and more than 1 nM, respectively (Table 1). These IC_{50} values for the IGF-I-enhanced DNA synthesis is more than 10-fold, 6-fold, or 20-fold above the IC_{50} for PI3-K, MEK, or $p70^{S6K}$, respectively.

IGF-I-induced phosphorylation of PKB/Akt via PI3-K in both attached and floating cells. To clarify the assumption that the IGF-I signal is mediated through PI3-K, activation of PKB/Akt by IGF-I was first examined using an antibody to phospho-PKB/Akt. Adding IGF-I to cells attached to the substratum caused phosphorylation of PKB/Akt within 15 min (Fig. 2). Phosphorylation of PKB/Akt by IGF-I was inhibited by pretreatment of cells with 10 μM of

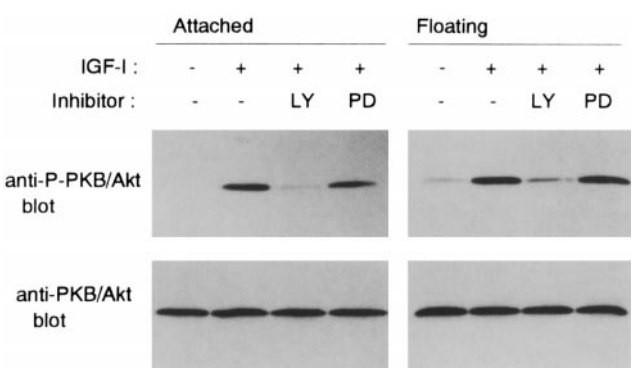

FIG. 2. Phosphorylation of PKB/Akt by IGF-I. Cells preincubated with or without 10 μM LY294002 (LY) or 50 μM PD98059 (PD) for 30 min were stimulated with 100 ng/ml of IGF-I for 15 min. Total cell lysates were electrophoresed, transferred onto membranes, and the blots were probed with antibodies to phospho-PKB/Akt (anti-P-PKB/Akt) or total PKB/Akt (anti-PKB/Akt).

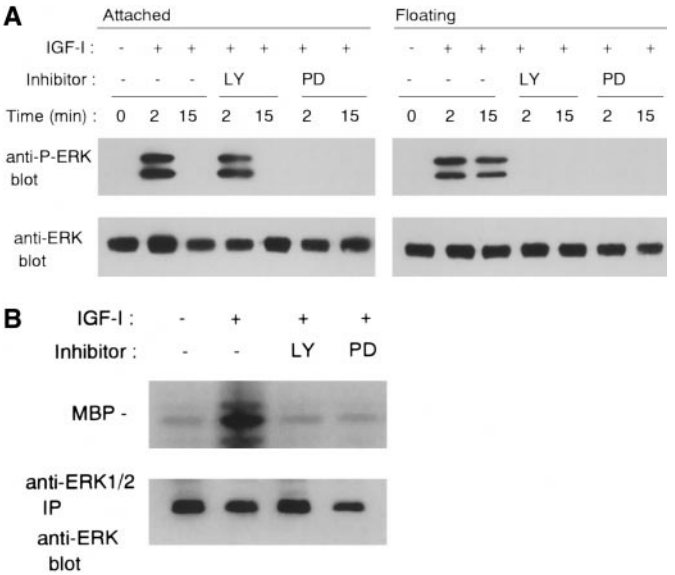

FIG. 3. Successive activation of ERK by IGF-I in floating MCF-7 cells. (A) Endogeneous phosphorylation of ERK by IGF-I. Cells preincubated with or without 10 μM LY294002 (LY) or 50 μM PD98058 (PD) for 30 min were stimulated with 100 ng/ml of IGF-I for 2 or 15 min. ERK was immunoprecipitated with anti-ERK antibody, and electrophoresed for Western blotting using antibody to phospho-ERK1/2 (anti-P-ERK) or total ERK (anti-ERK). (B) *In vitro* kinase assay for ERK in floating MCF-7 cells. ERK was immunoprecipitated from floating cells with anti-ERK antibody 15 min after adding IGF-I. Some of the immunoprecipitate was incubated with MBP as substrate, and the ^{32}P-labeled MBP (MBP) was electrophoresed and autoradiographed. The rest was electrophoresed, and Western blotted with anti-ERK antibody (anti-ERK).

LY294002, but not by 50 μM of PD98059. In floating cells, phosphorylation of PKB/Akt was induced by the addition of IGF-I (Fig. 2). The IGF-I-dependent phosphorylation of PKB/Akt in floating cells was inhibited by LY294002 (10 μM), but not by PD98059 (50 μM), as in attached cells.

Sustained activation of ERK in floating cells by IGF-I. Inhibition of IGF-I-induced DNA synthesis by MEK inhibitor PD98059 in anchorage-deficient cells, but not in attached cells, prompted us to examine whether the IGF-I signal is transduced from MEK to its known downstream signaling molecule ERK. Immunoprecipitation and western immunoblotting revealed that the addition of IGF-I to attached cells induced transient phosphorylation of ERK within 2 min, but not 15 min after incubation (Fig. 3A). The transient phosphorylation of ERK was resistant to PI3-K inhibitor LY294002 (10 μM), but susceptible to MEK inhibitor PD98059 (50 μM). Contrary to attached cells, ERK were successively phosphorylated in floating cells 2 and 15 min after adding IGF-I (Fig. 3A). The sustained phosphorylation of ERK in floating cells was blocked by either PD98059 (50 μM) or LY294002 (10 μM).

To confirm the sustained activation of ERK by IGF-I in floating cells, *in vitro* kinase assay for ERK was

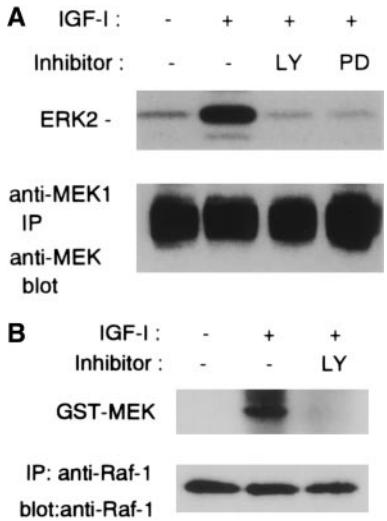

FIG. 4. Activation of MEK and Raf-1 by IGF-I in floating MCF-7 cells. (A) Induction of kinase activity of MEK by IGF-I. MEK1 was immunoprecipitated from floating cells, treated with or without 10 μM LY294002 (LY) or 50 μM PD98059 (PD) for 30 min followed by 100 ng/ml of IGF-I for 15 min, with anti-MEK1 antibody. The immunoprecipitated MEK1 was incubated with ERK2 as substrate (ERK2), or was electrophoresed for immunoblotting with anti-MEK1 antibody (anti-MEK). (B) Induction of kinase activity of Raf-1 by IGF-I. Immunoprecipitated Raf-1 with anti-Raf-1 antibody from floating cells, treated with or without 10 μM LY294002 for 30 min followed by 100 ng/ml of IGF-I for 15 min, was assayed for its *in vitro* kinase activity using GST-MEK as substrate (GST-MEK), or electrophoresed for Western blotting using anti-Raf-1 antibody (anti-Raf-1).

performed by immunoprecipitation of ERK from cells which were stimulated with IGF-I for 15 min with or without inhibitor. Incubation of ERK with MBP as substrate resulted in phosphorylation of MBP when ERK was immunoprecipitated from the IGF-I-stimulated floating cells (Fig. 3B), but not from attached cells (data not shown). Phosphorylation of MBP was not induced when cells were stimulated with IGF-I in the presence of either inhibitor LY294002 (10 μM) or PD98059 (50 μM) (Fig. 3B).

Activation of MEK and Raf-1 by IGF-I in floating cells. PI3-K functions in the Ras/ERK pathway involved in several receptor signaling pathways, and IGF-I stimulates PI3-K between Ras and Raf-1 (31), upstream of MEK (32, 33). Therefore, *in vitro* kinase assays for MEK and Raf-1 were performed to determine whether PI3-K is required for activation of MEK and Raf-1 by IGF-I. When the immunoprecipitated MEK from floating cells was incubated with ERK2 as substrate, phosphorylation of ERK2 was enhanced in the IGF-I-stimulated cells, but not in unstimulated cells (Fig. 4A). The enhanced phosphorylation of ERK2 by MEK *in vitro* was not observed when MEK was immunoprecipitated from floating cells which had been stimulated with IGF-I in the presence of either LY294002 (10 μM) or PD98059 (50 μM) (Fig. 4A).

Next, we examined whether Raf-1 is activated by IGF-I through PI3-K in floating cells, by means of incubating the immunoprecipitated Raf-1 with GST-MEK as substrate. The addition of IGF-I to floating cells induced the kinase activity of Raf-1 to phosphorylate GST-MEK (Fig. 4B). Pretreatment of floating cells with LY294002 (10 μM) before adding IGF-I caused a reduced phosphorylation of GST-MEK (Fig. 4B).

DISCUSSION

The addition of IGF-I to MCF-7 cells which were floating in medium significantly enhanced cellular DNA synthesis. The enhancement of DNA synthesis by IGF-I in anchorage-deficient cells was not due to a difference in the cell viability between the cultures with or without IGF-I, since the cell viability in floating cultures was comparable during incubation until the onset of DNA synthesis. These results imply that IGF-I does not act as a cell survival promoting factor such as in neuronal cells (34–36), but as a DNA synthesis-stimulating factor for anchorage-deficient MCF-7 cells. Pharmaceutical analyses using the enzyme inhibitors suggested that the IGF-I signal is mediated preferentially by PI3-K in attached cells to the substratum, and at least in part via both PI3-K and MEK in anchorage-deficient cells. The addition of IGF-I induced phosphorylation of PKB/Akt in both attached and floating cells. As phosphorylation of PKB/Akt accompanies the activation of its protein kinase activity (25, 37), it is suggested that PKB/Akt in both attached and floating cells is activated by IGF-I, and that this activation requires PI3-K. Western blotting revealed that ERK was phosphorylated transiently in adherent cells 2 min after stimulation with IGF-I and that the transient phosphorylation of ERK was not inhibited by PI3-K inhibitor. As recent findings demonstrated that activated PKB/Akt directly phosphorylates Raf-1 and inhibits ERK phosphorylation (38), it is suggested that the transient phosphorylation of ERK in adherent cells is regulated negatively, but not positively, by PI3-K through PKB/Akt. In contrast, ERK in floating cells was phosphorylated successively and its phosphorylation was inhibited by either inhibitors of PI3-K and MEK. Since the activation of ERK accompanies its kinase activity (39), the finding suggested that ERK is activated successively by IGF-I in floating cells, and that this activation requires both PI3-K and MEK. *In vitro* kinase assays for MEK and Raf-1, demonstrating that these kinases in floating cells were activated by IGF-I and that their activation was inhibited by PI3-K inhibitor, suggest that MEK and Raf-1 are activated by IGF-I in floating cells, and that the activation of both require PI3-K as an upstream signaling molecule.

The present findings demonstrate that the DNA synthesis-stimulatory signal of IGF-I is transduced via

PI3-K to PKB/Akt, but not to ERK, when cells are attached to the substratum. This supports previous findings (19). However, when the cells are anchorage-deficient, the IGF-I signal that stimulates cellular DNA synthesis is suggested to activate PI3-K, then activate two signaling pathways, leading to PKB/Akt or ERK. Signal transduction from PI3-K to ERK is observed in several receptor signaling pathways (31, 32, 40–42), whereas this result showing the anchorage-independent and IGF-I-dependent signaling from PI3-K to the ERK pathway is novel. The activation of dual signaling pathways may give cancerous cells an advantage in gaining the anchorage-independent growth property, since nonmalignant cells generally exhibit anchorage-dependent growth, and the growth signaling becomes blocked when they are prevented from attaching on a solid substratum (1, 2, 20).

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