Vol. 209, No. 1, 1995 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 6, 1995 Pages 131-138

EGF-INDUCED ACTIVATION OF 70-kDa S6 KINASE IN CHO CELLS EXPRESSING HUMAN EGF RECEPTORS

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Received February 23, 1995

We investigated epidermal growth factor (EGF)-induced activation of 85-kDa/110-kDa phosphatidylinositol (PI)-3-kinase and 70-kDa S6 kinase in Chinese hamster ovary cells expressing the human EGF receptor. EGF-induced activation of p70 S6 kinase was comparable to that induced by insulin, whereas that of PI-3-kinase in anti-phosphotyrosine immunoprecipitates was very small compared with insulin. Wortmannin, a p85/p110 PI-3-kinase inhibitor, inhibited EGF-induced activation of p70 S6 kinase in a dose-dependent manner. Given that several proteins homologous to catalytic subunit of p85/p110 PI-3-kinase have been identified and that wortmannin inhibits distinct form of PI-3-kinase, the present results suggest that wortmannin-sensitive kinases that resemble catalytic subunit of p85/p110 PI-3-kinase may participate in the signaling pathway from EGF receptors to p70 S6 kinase.

In propagation of the mitogenic signals initiated by many receptor tyrosine kinases such as those for epidermal growth factor (EGF), insulin, and platelet-derived growth factor (PDGF), phosphorylation of the 40S ribosomal protein S6 is an apparent prerequisite for activation of protein synthesis and cell growth (1). Growth factor-stimulated S6 phosphorylation is catalyzed by members of two families of growth factor-activated S6 serine-threonine kinases: 90-kDa S6 kinases (2) and 70-kDa S6 kinases (3,4). Although both families of S6 kinases are activated rapidly after addition of growth factors to quiescent cells, several studies indicate that p90 S6 kinase and p70 S6 kinase are regulated by distinct signaling pathways (5,6). The p90 S6 kinase is located downstream in the mitogen-activated protein (MAP) kinase cascade (7). On the other hand, it is controversial whether phosphatidylinositol (PI)-3-kinase, a heterodimeric enzyme consisting of 85-kDa regulatory subunit and 110-kDa catalytic subunit, is located in p70 S6 kinase signaling pathway (8-10). EGF induces an activation of p70 S6 kinase (11), whereas the effects of EGF on p85/p110

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The abbreviations used are: PI, phosphatidylinositol; MAP, mitogen-activated protein; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; CHO-ER cells, Chinese hamster ovary cells overexpressing human EGF receptors.

0006-291X/95 $5.00

PI-3-kinase activity appear variable (12-14). In order to determine whether p85/p110 PI-3-kinase is involved in EGF-induced p70 S6 kinase activation pathway or not, we investigated EGF-induced activation of p85/p110 PI-3-kinase in anti-phosphotyrosine immunoprecipitates and p70 S6 kinase in Chinese hamster ovary cells expressing human EGF receptors (CHO-ER cells).

MATERIALS AND METHODS

Materials: Mouse EGF was obtained from Takara Shuzo (Kyoto, Japan). A stock solution (1mM) of wortmannin, kindly provided by Dr. Y. Matsuda (Kyowa Hakko Kogyo Co., Machida, Japan), was prepared in Me_2SO, stored at -20°C in the dark, and diluted with incubation medium immediately before use; the final concentration of Me_2SO in the medium was <0.1%.

Cell culture: CHO-ER cells were maintained in Ham's F-12 medium supplemented with 10% fetal bovine serum (15). Confluent cells were further cultured in serum-free Ham's F-12 for 16 h. Quiescent cells were left untreated or treated with wortmannin for 10 min prior to stimulation with EGF or insulin.

Kinase assays: The assay of p70 S6 kinase was performed as described by Flotow and Thomas (16). Cell lysates were incubated with anti-p70 S6 kinase antibodies complexed with protein A-Sepharose (Zymed, So. San Francisco, CA). Antibodies to p70 S6 kinase were raised against synthetic peptides corresponding to predicted amino acid residues 2 to 23 of the kinase (4). The immunoprecipitates were incubated in 10 μl of a solution containing 50 mM MOPS (pH 7.2), 1 mM dithiothreitol, 10 mM p-nitrophenylphosphate, 0.05% Triton X-100, 50 μM phenylmethylsulfonyl fluoride, 5 mM $MgCl_2$, 100 μM ATP, 3 μCi of [γ-^{32}P]ATP (~4000 Ci/mmol, ICN, Irvine, CA), and a peptide (0.1 mM) corresponding to amino acids 230 to 249 of the 40S ribosomal protein S6 as substrate (16). The apparent Km and Vmax for phosphorylation of this peptide by p70 S6 kinase are similar to those obtained for S6 within the ribosome (16). After 30 min at 37°C, portions of reaction mixture were withdrawn and the reaction was terminated by the addition of EDTA and adenosine to final concentrations of 20 and 1.5 mM, respectively. The reaction mixtures were spotted onto P81 paper (Whatman, Maidstone, United Kingdom) and ^{32}P incorporation into peptides was measured.

PI-3-kinase activity was measured in immunoprecipitates with anti-phosphotyrosine antibodies (ICN Biochemicals, Costa Mesa, CA) complexed with protein G-Sepharose (Pharmacia, Uppsala, Sweden) (17). The immunoprecipitates were suspended in a solution containing 20 mM Hepes (pH 7.1), 10 mM NaCl, PI (200 μg/ml), 50 μM ATP, 5 μCi of [γ-^{32}P]ATP, and 10 mM $MgCl_2$. After incubation for 6 min at room temperature, the reaction was stopped by adding 0.3 volume of 4M HCl, followed by 2 volumes of chloroform:methanol (1:1). After centrifugation, the lower organic phase was analyzed by thin-layer chromatography on silica gel plates. Radioactivity on the dried plate was visualized after development with a Bioimaging Analyzer.

RESULTS AND DISCUSSION

EGF-induced activation of p70 S6 kinase and p85/p110 PI-3-kinase in CHO-ER cells

We first examined the kinetics of p70 S6 kinase activation in CHO-ER cells, because EGF induces a biphasic activation of p70 S6 kinase in Swiss 3T3 cells (11). Since parent CHO cells showed no response in p70 S6 kinase activity to 10 and 100 nM EGF, we could observe the signaling properties through wild-type EGF receptors expressed in CHO cells. EGF (1 nM) induced a monophasic activation of p70 S6 kinase in CHO-ER cells; activity was increased after exposure of cells to EGF for 5 min, peaked (fourfold incrase) at 10 min, and subsequently decreased (Fig. 1). Thus, CHO-ER cells did not show the late phase of

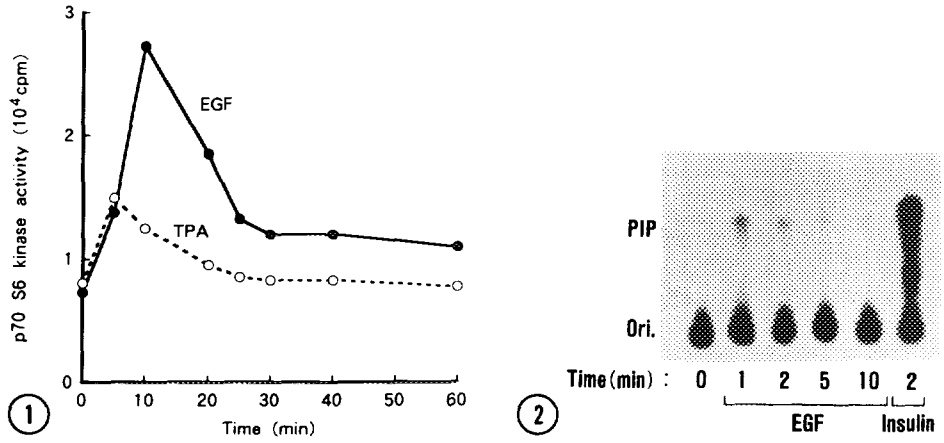

Fig. 1. EGF-induced activation of p70 S6 kinase. CHO-ER cells were incubated with 1nM EGF or 100 ng/ml 12-O-tetradecanoyl phorbol-13-acetate (TPA) for the indicated times, and cell extracts were prepared and assayed for p70 S6 kinase activity as described in Materials and Methods. Results are representative of three separate experiments.

Fig. 2. EGF-induced activation of PI-3-kinase in anti-phosphotyrosine immunoprecipitates. CHO-ER cells were incubated with 10 nM EGF or 100 nM insulin for the indicated times, and cell extracts were prepared and assayed for PI-3-kinase activity in anti-phosphotyrosine immunoprecipitates. Results are representative of two separate experiments.

kinase activation in response to EGF that is apparent in Swiss 3T3 cells. Under our assay conditions, 1 nM EGF induced a biphasic activation of p70 S6 kinase in Swiss 3T3 cells, with the first peak at 10 min and the second peak at 40 min (data not shown). 12-O-Tetradecanoyl phorbol-13-acetate also induced a small and transient activation of p70 S6 kinase in CHO-ER cells, with a peak of activity after 5 min of stimulation (Fig. 1).

We then examined the kinetics of activation of p85/p110 PI-3-kinase. A small activation of PI-3-kinase in anti-phosphotyrosine immunoprecipitates occured rapidly after exposure of cells to 10 nM EGF, reaching a maximum after 1-2 min of stimulation, and subsequently decreased (Fig. 2). Exposure of cells to 100 nM insulin for 2 min caused a marked increase in PI-3-kinase activity in anti-phosphotyrosine immunoprecipitates (Fig. 2). Regulatory subunit of p85/p110 PI-3-kinase has been shown to bind directly to activated receptors and receptor substrates at domains that are phosphorylated on tyrosine and contain the Tyr-X-X-Met motif (where X is any amino acid) (18-20). In insulin-stimulated cells, insulin receptor substrate 1 which possesses several tyrosine-phosphorylated Tyr-X-X-Met motif (21) is a major tyrosine phosphorylated protein to which p85/p110 PI-3-kinase binds. On the other hand, the EGF receptor lacks a tyrosine-phosphorylated Tyr-X-X-Met motif. Recent reports have shown that p85/p110 PI-3-kinase binds to ErbB-3 which possesses tyrosine-phosphorylated Tyr-X-X-Met motif (22,23). In addition, ErbB-3 is involved in activation of p85/p110 PI-3-kinase by EGF in certain cells such as A431 cells (24). Thus, ErbB-3 might be a candidate for tyrosine phosphorylated protein to which p85/p110 PI-3-kinase binds, although it remains unclear at this point that ErbB-3 actually interact with EGF

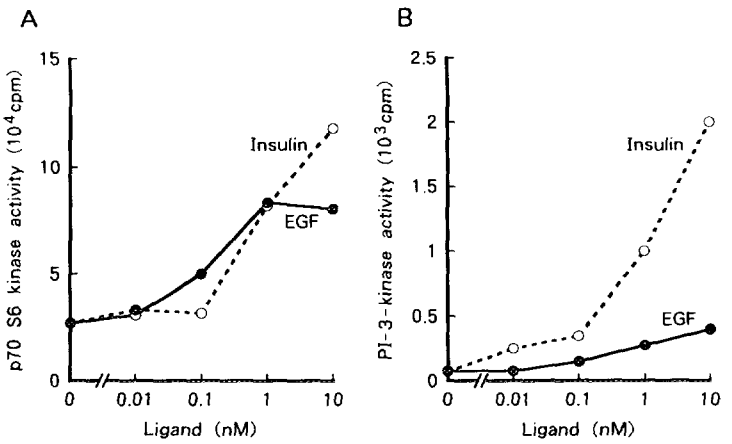

Fig. 3. Activation of p70 S6 kinase and PI-3-kinase in response to EGF and insulin. (A) CHO-ER cells were incubated with EGF or insulin for 10 min and p70 S6 kinase activity was measured. (B) CHO-ER cells were incubated with EGF or insulin for 2 min and PI-3-kinase activity in anti-phosphotyrosine immunoprecipitates was measured. Results are representative of three separate experiments.

receptors in CHO-ER cells. On the basis of these results, we incubated CHO-ER cells with EGF for 10 or 2 min in subsequent assays for p70 S6 kinase or p85/p110 PI-3-kinase, respectively.

Activation of p70 S6 kinase and p85/p110 PI-3-kinase by EGF and insulin

After exposure of CHO-ER cells to various concentrations of EGF and insulin, p70 S6 kinase and p85/p110 PI-3-kinase activities were measured. Both EGF and insulin increased p70 S6 kinase activity in a dose-dependent manner. EGF-induced p70 S6 kinase activation was apparent at 0.1 nM and reached a maximum at 1 to 10 nM, whereas higher concentration (1 nM) of insulin was needed to cause an apparent activation of p70 S6 kinase (Fig. 3A). Although EGF induced a two- to threefold increase in PI-3-kinase activity in anti-phosphotyrosine immunoprecipitates, such an increase was very small when compared with that induced by insulin (Fig. 3B). Although 10 nM EGF increased p70 S6 kinase activity comparable to that induced by 1 nM insulin, EGF (10 nM)-induced PI-3-kinase activity in anti-phosphotyrosine immunoprecipitates was similar to that induced by 100 pM insulin which failed to increase p70 S6 kinase activity (Fig. 3A and B).

Insulin has been shown to activate p85/p110 PI-3-kinase (25,26), whereas the effects of EGF on p85/p110 PI-3-kinase activity appear variable. When PI-3-kinase activity was measured in anti-phosphotyrosine immunoprecipitates, EGF activates PI-3-kinase very weakly compared with insulin and insulin-like growth factor-1 in KB cells (14), although EGF induced a rapid and more than 15-fold increase in PI-3-kinase activity in PC12 cells (12,13). In CHO-ER cells, EGF-induced activation of PI-3-kinase associated with tyrosine phosphorylated proteins is very small compared with insulin, suggesting that p85/p110 PI-3-kinase is not located in p70 S6 kinase signaling pathway through EGF receptors. In addition, it seems unlikely that small increase in p85/p110 PI-3-kinase activity is sufficient to induce full activation of p70 S6 kinase, because insulin failed to increase p70 S6 kinase activity at a

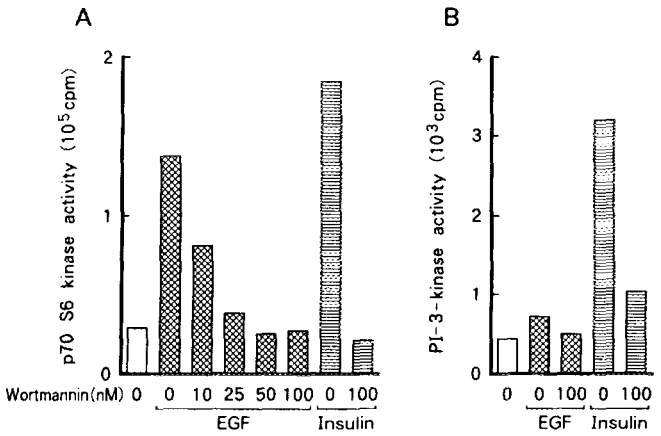

Fig. 4. Effect of wortmannin on EGF and insulin-induced activation of p70 S6 kinase and PI-3-kinase. CHO-ER cells were exposed to wortmannin for 10 min and then incubated in the absence or presence of 10 nM EGF or 100 nM insulin. Cells were lysed and p70 S6 kinase activity (A) and PI-3-kinase activity in anti-phosphotyrosine immunoprecipitates (B) were measured. Results are representative of five separate experiments.

concentration where PI-3-kinase activity associated with tyrosine phosphorylated proteins was increased comparable to that induced by 1-10 nM EGF.

Effect of wortmannin on EGF- and insulin-induced activation of p70 S6 kinase

Since wortmannin is shown to inhibit p85/p110 PI-3-kinase activity possibly by binding to the catalytic subunit of the enzyme (27), we investigated the effect of wortmannin on EGF- and insulin-induced activation of p70 S6 kinase and p85/p110 PI-3-kinase. Wortmannin inhibited EGF-induced p70 S6 kinase activation in a dose-dependent manner (Fig. 4A); half-maximal inhibition was apparent at ~10 nM wortmannin. Wortmannin (100 nM) completely abolished p70 S6 kinase activation induced by both EGF and insulin (Fig. 4A). Wortmannin (100 nM) inhibited the EGF-induced increase in PI-3-kinase activity in the anti-phosphotyrosine immunoprecipitates of CHO-ER cells, whereas the insulin-induced activation of PI-3-kinase was inhibited by only ~70%. This level of PI-3-kinase activity in anti-phosphotyrosine immunoprecipitates was much higher than that induced by 10 nM EGF stimulation (Fig. 4B).

In contrast, wortmannin, at up to 500 nM, exerted little effect on EGF-induced activation of MAP kinase (data not shown). EGF induces an activation of Ras (28) and Ras is implicated in signaling pathway from EGF receptors to MAP kinase (7). A recent report has shown that Ras interacts directly with the catalytic subunit of p85/p110 PI-3-kinase and regulates p85/p110 PI-3-kinase activity (29). However, dominant negative Ras mutant with serine to asparagine sustitution at amino acid 17 blocks activation of MAP kinase but not p70 S6 kinase (10). In addition, we failed to detect PI-3-kinase activity in anti-Ras (Y13-259 and

Y13-238) immunoprecipitates of EGF-treated CHO-ER cells (data not shown). Taken together, p70 S6 kinase signaling pathway seems to be independent of Ras.

Using wortmannin and LY294002, a competitive inhibitor of the ATP binding site specific for p85/p110 PI-3-kinase, recent reports (8,9) have suggested that p85/p110 PI-3-kinase is located in p70 S6 kinase signaling pathway induced by insulin and PDGF. Cheatham et al. (8) have shown that LY294002 inhibits insulin-induced activation of p85/p110 PI-3-kinase and p70 S6 kinase without affecting insulin-stimulated MAP kinase and p90 S6 kinase in 3T3-L1 adipocytes. Chung et al. (9) have also shown that wortmannin inhibits PDGF- and insulin-dependent activation of p70 S6 kinase in HepG2 cells. However, the possibility that these inhibitors affect other lipid kinases than p85/p110 PI-3-kinase is not totally eliminated. p85/p110 PI-3-kinase-related proteins are identified in yeast ; TOR2 is related to the catalytic subunit of bovine PI-3-kinase (30) and Vps34, a vacuolar sorting protein, has PI-3-kinase activity (31). Rapamycin is shown to act on TOR2 and inhibit activation of p70 S6 kinase (32,33). Recently, a mammalian rapamycin-binding protein is identified and shown to be highly related to TOR1 and TOR2 (34). These observations suggest that mammalian TOR may be implicated in p70 S6 kinase signaling, although it is not yet known whether TOR possesses PI-3-kinase activity. In addition, distinct form of PI-3-kinase activity which is specifically activated by G protein $\beta\gamma$ subunit is identified and this enzyme is shown to be sensitive to wortmannin (35). Furthermore, it is suggested that there are at least eight proteins which resemble catalytic subunit of p85/p110 PI-3-kinases in mammalian cells and three of them form stable complex with receptor tyrosine kinases (36). Taken together, it is possible that distinct form of PI-3-kinases which do not associate with tyrosine-phosphorylated proteins are responsible for mediating the stimulation of p70 S6 kinase activation.

More recently, Ming et al. (10) have shown that PDGF induces activation of p70 S6 kinase but not receptor-associated p85/p110 PI-3-kinase activity in PAE cells expressing mutant PDGF receptors which lack one of p85/p110 PI-3-kinase binding sites. In addition, insulin-induced activation of p70 S6 kinase is unaltered in Chinese hamster ovary cells co-expressing human insulin receptors and a deletion mutant of the p85 subunit of PI-3-kinase, in which the insulin-induced increase in p85/p110 PI-3-kinase activity is markedly reduced[1]. These observations indicate that the heterodimeric p85/p110 PI-3-kinase might not be the molecule responsible for activating the p70 S6 kinase pathway. Taken together, our results suggest the possibility that wortmannin-sensitive kinases that resemble a catalytic domain of p85/p110 PI-3-kinase may participate in the signaling pathway from EGF receptors to p70 S6 kinase.

ACKNOWLEDGMENTS

We thank Drs. H. A. Lane and G. Thomas for anti-p70 S6 kinase antibodies and ribosomal protein S6, Dr. M. Shibuya for human EGF receptor cDNA, and Dr. Y. Matsuda for wortmannin.

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[1]Hara, K., Yonezawa, K., Sakaue, H., Kotani, K., Kotani, K., Kojima, A., Waterfield, M. D., and Kasuga, M. manuscript submitted.

This work was supported by a grant to Y. O., and by a grant-in-aid for Cancer Research to M. K., from the Ministry of Education, Science, and Culture of Japan.

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