Phosphatidylinositol 3'-Kinase-independent p70 S6 Kinase Activation by Fibroblast Growth Factor Receptor-1 Is Important for Proliferation but Not Differentiation of Endothelial Cells*

(Received for publication, December 3, 1996, and in revised form, June 10, 1997)

Shigeru Kanda‡, Matthew N. Hodgkin§, Richard J. Woodfield§, Michael J. O. Wakelam§, George Thomas§, and Lena Claesson-Welsh|  

From the Ludwig Institute for Cancer Research, Biomedical Centre, Box 595, S-751 24 Uppsala, Sweden, §CRC Institute for Cancer Studies, Queen Elizabeth Hospital, Birmingham B15 2TH, United Kingdom, and ¶Friedrich-Miescher Institute, P. O. Box 2543, CH-4002 Basel, Switzerland

p70(e6k) has a role in cell cycle progression in response to specific extracellular stimuli. The signal transduction pathway leading to activation of p70(e6k) by fibroblast growth factor receptor-1 (FGFR-1) was examined in FGF-2-treated rat L6 myoblasts. p70(e6k) was activated in a biphasic and rapamycin-sensitive manner. Although phosphatidylinositol 3'-kinase was not activated in the FGF-2 treated cells, as judged from in vitro and in vivo analyses, wortmannin and LY294002 treatment inhibited p70(e6k) activation. Inhibition of protein kinase C (PKC), by bisindolylmaleimide or by chronic phorbol ester treatment of the FGFR-1 cells, suppressed but did not block p70(e6k) activation. In cells expressing a point-mutated FGFR-1, Y766F, unable to mediate PKC activation, p70(e6k) was still activated, in a bisindolylmaleimide- and phorbol ester-resistant manner. The involvement of S6 kinase in FGFR-1-dependent biological responses was examined in murine brain endothelial cells. In response to FGF-2, these cells differentiate to form tube-like structures in collagen gel cultures and proliferate when cultured on fibronectin. p70(e6k) was not activated in endothelial cells on collagen, whereas activation was observed during proliferation on fibronectin. In agreement with this finding, rapamycin inhibited the proliferative but not the differentiation response. Our results indicate that FGFR-1 mediates p70(e6k) activation by a phosphatidylinositol 3'-kinase-independent mechanism that does not require PKC activation and, furthermore, proliferation, but not differentiation of endothelial cells in response to FGF-2, is associated with p70(e6k) activation.

The p70 S6 kinase (p70(e6k)) is a serine/threonine kinase that phosphorylates 40 S ribosomal protein S6, in response to a number of extracellular stimuli (1, 2). The two isoforms of p70(e6k), the 70-kDa s6k I (cytosolic form) and the 85-kDa s6k II (nuclear form), are derived from alternatively spliced products (3, 4) from a single gene (3). Extracellular stimuli induce acute phosphorylation on multiple serine and threonine residues within p70(e6k), which are associated with its activation. Four of these residues are located in the carboxyl terminus of p70(e6k) (5). These sites are potential mitogen-activated protein kinase targets. However, mitogen-activated protein kinase fails to activate p70(e6k) after phosphorylation of these sites in vitro (6), and p70(e6k) activation lies on a Ras-independent pathway (7, 8). Protein kinase C (PKC), phosphatidylinositol 3-kinase (PI3-kinase), and protein kinase B have been implicated as upstream signaling molecules of p70(e6k) activation in insulin-, platelet-derived growth factor (PDGF)-, epidermal growth factor (EGF)-, and interleukin-2-treated cells (9). Recently, p70(e6k) was moreover shown to complex with and to be activated by the GTP-binding Rho family proteins Rac1 and Cdc42 (10). However, the relative contribution of these pathways to activation of p70(e6k) is unclear (8).

Rapamycin is a potent and specific inhibitor of p70(e6k), preventing phosphorylation and activation of p70(e6k) by all known external stimuli (11–15). After binding of rapamycin to its cellular receptor, the FK506-binding protein-12 (FKBP-12), this complex targets TOR kinases in Saccharomyces cerevisiae or the related protein FKBP-12-rapamycin-associated protein/rapamycin-FKBP target 1/mammalian TOR in mammalian cells (16–18). Inactivation of p70(e6k) by rapamycin is associated with selective dephosphorylation of a unique set of serine and threonine sites, flanked by large aromatic residues, in p70(e6k) (19). Rapamycin is known to inhibit growth of many types of cells; causing G1 arrest in T lymphocytes and delaying entry into S phase in fibroblasts (11, 12). Microinjection of a neutralizing antibody against p70(e6k) or p85(e6k) has also been shown to block the entry into S phase of injected cells (20, 21). These results indicate that p70(e6k) activation is important for cell cycle progression.

Fibroblast growth factors (FGF) are heparin-binding polypeptide growth factors, which form a family of nine members (22). Extracellular signaling by FGFs is transduced via specific receptor tyrosine kinases, denoted FGF receptor-1 to -4 (23, 24). Heparin and heparan sulfate proteoglycans are known to modulate ligand binding to the receptor tyrosine kinase. Binding of FGFs to the receptor tyrosine kinase leads to receptor

This paper is available on line at http://www.jbc.org

© 1997 by The American Society for Biochemistry and Molecular Biology, Inc.
Printed in U.S.A.
dimerization and activation of the kinase domain, followed by autophosphorylation of the receptor and association with downstream signaling components. Thus far, only one Src homology 2 (SH2) domain-containing protein, phospholipase C-γ (PLC-γ), has been shown to bind directly to FGF receptor-1, via a carboxyl-terminal autophosphorylation site at Tyr-766 in the receptor (25). The FGF receptors are known to mediate a variety of cellular responses, such as cell proliferation, migration, and differentiation (23, 24). We have previously shown that murine capillary endothelial cells respond to FGF-1 and FGF-2 treatment either by proliferation or by differentiation, the latter visualized in vitro as tube formation of cells cultured in collagen gels (26). It is likely that several distinct signal transduction pathways, coupling directly or indirectly to the receptor, contribute to establish these responses. p70s6k has been shown to be activated in FGF-treated cells (27). In this paper, we have used different inhibitors of signal transduction pathways, known to contribute to p70s6k activation, as well as a mutant FGF receptor unable to bind PLC-γ, to characterize FGF-induced p70s6k activation biochemically and to investigate its function in cellular responses to FGF.

MATERIALS AND METHODS

Cell Culture—Rat L6 myoblasts expressing wild-type FGF receptor-1 and Y766F point-mutated FGF receptor-1 (28) were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Life Technologies, Inc., London, UK) supplemented with 10% fetal bovine serum, at 37 °C. The IBE cell line is a capillary endothelial cell line established from H-2Kb-tsA58 donor, UK (29) and differentiated in parallel. IBE cells were cultured routinely in Ham’s F12 medium containing 20% heat-inactivated fetal bovine serum. Dependent on the assay, cell lines were cultured on 6-cm dishes, which were incubated at 33 °C. The culture medium was replaced by DMEM containing 1% Nonidet P-40 on ice. Collected cell lysate was frozen in liquid nitrogen and kept at −80 °C until assay. After thawing, the lysate was centrifuged at 10,000 × g for 30 min and then 100 μg of lysed protein was incubated with the anti-p70s6k antisemur M5 (3) at 4 °C for 2 h, followed by precipitation with Protein A-Sepharose beads. Beads were washed 3 times with EB containing Nonidot P-40, once with dilution buffer (DB), 50 mM MOPS, pH 7.2, 5 mM MgCl2, 0.2% Triton X-100, 1 mM dithio- reitol) and resuspended in 5 μl of EB. The kinase reaction was initiated by addition of 5 μl of DB containing 200 μM ATP, 10 mM 4-nitrophenyl phosphate, 10 μg of 40 S ribosomal protein, and 3 μCi of [γ-32P]ATP to the beads. The reaction proceeded at 37 °C for 30 min and was stopped by addition of SDS sample buffer, followed by electrophoresis in 15% SDS-polyacrylamide gels. After electrophoresis, the gel was fixed and dried and then exposed on an Image Analyzer (Fuji). Pie-3 Kinase in Vitro Assay—Cells kept in DMEM containing 0.5% FBS, overnight, were either unstimulated or stimulated with 100 ng/ml FGF-2 or platelet-derived growth factor-BB (PDGF-BB) for indicated times, rinsed, and then lysed in 25 mM HEPES, pH 7.5, 150 mM NaCl, 0.1 mM sodiu m orthovanadate, 10% glycerol, 1% Nonidot P-40, 5 mM EDTA, 100 mM/mL aprotinin, and 1 mM PMSF. Clarified cell lysates were immunoprecipitated with anti-phosphotyrosine monoclonal antibody, PY-20 (Transduction Laboratory, Lexington, KT). Protein A-Sepharose immune complexes were washed twice with phosphate-buffered saline containing 1% Nonidot P-40, once with phosphate-buff ered saline, once with 0.1 M Tris-HCl, pH 7.5, containing 0.5 M LiCl, once with distilled water, and once with 25 mM HEPES, pH 7.5, 100 mM NaCl, 1 mM EDTA. The beads were suspended in 50 μl of 25 mM HEPES, pH 7.5, 100 mM NaCl, 0.5 mM EGTA, and 10 μg of phosphaty- dylinositol (sonicated for 5 min at 20 °C; Sigma) and preincubated at 20 °C for 10 min. Twenty μCi of [γ-32P]ATP and MgCl2 (final concentration, 10 μM) were added, and samples were further incubated for 10 min at 20 °C. The reaction was stopped by addition of chloroform/methanol (v/v) (200 μl) and 1 mM PMSF. Phospholipids were extracted with chloroform, and the organic phase was washed with methanol, 1 mM HCl (1:1). Reaction products were detected in vacuo, dissolved in chloroform, spotted on silica Gel-60 plates (Merck) impregnated with 1% potassium oxalate, and resolved by chromatography in chloroform/methanol, 28% ammonia/water (43:38:5:7) for 45 min. Phosphorized lipids were extracted into chloroform/methanol and dried in vacuo. Products were detected by Image Analyzer (Fuji) and then exposed on x-ray film (Fuji). PIE-3 Kinase in Vivo Assay—L6 cells expressing FGF-1 were washed with phosphate-free DMEM containing 0.1% fatty acid-free BSA, 0.0375% sodium bicarbonate, and 20 mM HEPES, pH 7.4, and labeled for 90 min in medium containing 300 μCi/ml of [32P]Pi. Washed cells were stimulated with FGF-2 for 5 min, and the lipids were extracted into chloroform/methanol, deacylated using monomethylamine, and deglycerated using periodate as described (30). The generated inositol phosphates (which corresponded to the inositol lipids) were separated by anion exchange high performance liquid chromatography on a 25-μm partisphere SAX column, eluted with a linear gradient of ammonium dihydrogen phosphate (0.5 μl, pH 3.8) at 1 μl/min over 110 min. Fractions were collected every 0.05 min and [32P] determined by scintillation counting; peak retention times were compared with authentic labeled standards, which were run every 5th injection. Diacylglycerol (DAG) Kinase-Linked Assay—Changes in DAG mass were determined by the DAG kinase-linked assay as described (31). Briefly, cells were stimulated as above; the medium was aspirated, and incubations were terminated by the addition of ice-cold methanol. The lipids were extracted into chloroform/methanol and dried in vacuo. The solution of lipids were then resuspended with 0.5% BSA in cell cytosol DAG kinase and [γ-32P]ATP, the generated [32P]Phosphatidate was separated by thin layer chromatography and analyzed using a PhosphorImager, and the mass of DAG was determined by comparison to a standard curve generated in parallel.

Cell Proliferation Assay—Mouse brain capillary endothelial cells (IBE cells) were inoculated into 24-well culture plates coated with x-ray film (Fuji). p70 s6 Kinase Activity Assay—The p70s6k activity assay was performed as described previously (3) with some modifications. L6 cells cultured on 6-cm dishes were either unstimulated or stimulated with 100 ng/ml ligand for 10 or 60 min. IBE cells were inoculated on either fibronectin- or collagen-coated dishes and maintained in 1% Nonidot P-40 containing 0.25% BSA, cultured for 4 h at 33 °C, and then stimulated with 100 ng/ml FGF-2, for indicated times. Cells were washed with extraction buffer (EB), which is composed of 50 mM Tris-HCl, pH 8.0, 120 mM NaCl, 20 mM NaF, 1 mM benzamidine, 1 mM EDTA, 6 mM EGTA, 15 mM Na3P04, 50 mM 4-nitrophenyl phosphate, 0.1 mM PMSF. After washing, cells were lysed in EB containing 1% Nonidot P-40 on ice. Collected cell lysate was frozen in liquid nitrogen and kept at −80 °C until assay. After thawing, the lysate was centrifuged at 10,000 × g for 30 min and then 100 μg of lysed protein was incubated with the anti-p70s6k antisemur M5 (3) at 4 °C for 2 h, followed by precipitation with Protein A-Sepharose beads. Beads were washed 3 times with EB containing Nonidot P-40, once with dilution buffer (DB), 50 mM MOPS, pH 7.2, 5 mM MgCl2, 0.2% Triton X-100, 1 mM dithio- reitol) and resuspended in 5 μl of EB. The kinase reaction was initiated by addition of 5 μl of DB containing 200 μM ATP, 10 mM 4-nitrophenyl phosphate, 10 μg of 40 S ribosomal protein, and 3 μCi of [γ-32P]ATP to the beads. The reaction proceeded at 37 °C for 30 min and was stopped by addition of SDS sample buffer, followed by electrophoresis in 15% SDS-polyacrylamide gels. After electrophoresis, the gel was fixed and dried and then exposed on an Image Analyzer (Fuji).
Human p70s6k activity was seen in the wild-type and mutant FGF treatment. However, at 60 min, similar induction (250–300%) was observed. In the mutant Y766F receptor expressing cells (Fig. 1A), the level of p70s6k activity was comparatively lower at 10 min than at 60 min. Culture was continued for additional 3.5 h, at which point the medium was refreshed and the second layer of collagen was added onto the cells. After gelation of the second layer of collagen, the cells were cultured overnight. Photographs were taken under phase-contrast microscopic examination.

**RESULTS**

**FGF Receptor-1 Transduces Signals for Activation of p70s6k**

p70s6k is known to be activated in response to various mitogens, including insulin, PDGF, EGF, and serum (9). Agonist-stimulated activation of p70s6k is biphasic, with a rapid transient peak, followed by a sustained plateau (27, 32). The relevance of the first peak is not clear, but the sustained phase is thought to represent biological activity (32). Several distinct signal transduction pathways have been implicated in p70s6k activation. We used transfected L6 myoblasts, expressing either the wild-type FGF receptor-1, or a point-mutated FGF receptor-1, Y766F, to examine signal transduction pathways leading to p70s6k activation in response to FGF.

Fig. 1A shows that wild-type and Y766F FGF receptor-1-transfected L6 cells expressed similar levels of receptors and that the receptors responded to ligand stimulation with induction of tyrosine kinase activity. In contrast, the parental cells lack detectable expression of FGF receptors, and FGF-2 stimulation of the untransfected parental cells failed to induce p70s6k activation (data not shown). p70s6k activation was examined by immunoprecipitation of p70s6k, from unstimulated and FGF-2-stimulated cells, followed by incubation in kinase buffer and [γ-32P]ATP, in the presence of 40 S ribosomes, serving as a substrate for the immunoprecipitated p70s6k. After SDS-PAGE of the samples, 32P-labeled 40 S ribosomal protein s6 was quantified using a PhosphorImager Analyzer. p70s6k activation was analyzed after 10 and 60 min of FGF-2 treatment, to measure early and sustained phases, respectively. Fig. 1B shows that p70s6k was activated in a sustained manner in FGF-2-stimulated L6 cells expressing the wild-type FGF receptor-1. In the mutant Y766F receptor-1 transfected cells expressing FGF-2 for 5 or 30 min, the level of p70s6k activity was comparatively lower at 10 min treatment. However, at 60 min, similar induction (250–300%) of p70s6k activity was seen in the wild-type and mutant FGF receptor-1 expressing cells. These results indicate that signals for p70s6k activation can be transduced via FGF receptor-1 and that the major autophosphorylation site, Tyr-766, which is required for binding and activation of PLC-γ, is not obligatory for FGF receptor-1-mediated p70s6k activation.

**Effects of Signal Transduction Pathway Inhibitors on p70s6k Activation**

Different signal transduction pathways have been implicated in p70s6k activation, and inhibition of the functions of PI3-kinase and PKC have been used to demonstrate roles for pathways involving these enzymes. We analyzed the effects of rapamycin, wortmannin (PI3-kinase inhibitor), and bisindolylmaleimide (PKC inhibitor) in FGF-2-induced p70s6k activation. In addition, chronic treatment of cells with PMA was used to down-regulate PKC. Fig. 2 shows that treatment of the wild-type FGFR-1 expressing L6 cells with either of these four different drugs attenuated FGF-2-induced p70s6k activation, both at 10 and 60 min. Rapamycin failed to bring the activity of p70 down to basal, even at the relatively high dose of 100 ng/ml. Treatment of cells with another PI3-kinase inhibitor, LY294002, brought p70s6k activity down to the basal level when used at concentration of 30 μM (data not shown).

We have previously shown that activation of FGF receptor-1, expressed in porcine aortic endothelial cells, does not lead to activation of PI3-kinase in vitro (33). In agreement, we failed to detect activation in vitro of PI3-kinase in L6 myoblasts expressing FGF receptor-1 (Fig. 3). The experiment was performed using L6 myoblasts expressing FGF receptor-1, which were stimulated for 5 or 30 min with FGF-2. As a positive control, PDGF-BB was used to stimulate PDGF receptors, endog-
PI3-kinase for immunoprecipitation (data not shown).

Furthermore, analysis of in vivo $^{32}$P-labeled L6 cells expressing FGR-1 showed that FGF-2 also did not stimulate PI3-kinase activity as determined by changes in $^{3}$-phosphorylated lipids (Table I). The table shows that FGF-2 stimulated significant turnover of inositol phospholipids with a greater than 2-fold increase in the radioactivity incorporated into PIP within 5 min and an approximate 50% increase in radioactivity associated with PI(4,5)P$_2$. There was a small increase in radioactivity associated with PIP$_2$; however, the level of PIP$_3$ is extremely low and the apparent change is probably due to an increase in basal PI3-kinase activity acting upon the elevated $^{32}$Pi/PI(4,5)P$_2$; indeed, the radioactivity associated with PIP$_3$ compared with PIP$_2$ remains constant at approximately 0.03%. These data strongly imply that inhibition by wortmannin of p70$^{s6k}$ activation in the FGF-2-stimulated cells did not involve the classical PI3-kinase.

Role of PKC in p70$^{s6k}$ Activation—The Y766F FGF receptor-1 mutant lacks the ability to mediate phosphorylation and activation of PLC-$\gamma$. Active PLC-$\gamma$ hydrolyzes PIP$_2$ to inositol 1,4,5-trisphosphate and diacylglycerol (DAG), leading to intracellular Ca$^{2+}$ fluxes and activation of PKC, respectively. In the Y766F mutant FGF receptor-1 expressing cells, PKC could potentially still be activated in response to FGF-2, via PLC-$\gamma$-independent DAG formation. We therefore analyzed formation of DAG, by use of a DAG kinase-linked assay, on FGF-2-stimulated wild-type or Y766F FGR-1 expressing L6 cells (Table II). In the wild-type receptor expressing L6 cells DAG formation was increased approximately 2-fold, in response to FGF-2, at 10 and 60 min of stimulation. A similar level of DAG formation was increased approximately 2-fold, in response to FGF-2-stimulated wild-type or Y766F receptor expressing cells (Table II). In the wild-type receptor expressing L6 cells DAG formation did not increase in response to FGF-2 stimulation (Table II).

As shown in Fig. 1B, the extent of p70$^{s6k}$ activity at the mitogenically relevant, sustained phase was very similar in wild-type FGF receptor-1 and Y766F mutant receptor expressing cells. Moreover, Fig. 4 shows that the level of p70$^{s6k}$ activity in the Y766F mutant receptor expressing cells was not affected by treatment with bisindolylmaleimide, to inhibit PKC, or chronic exposure to PMA, to deplete cellular PKC levels. In these cells, rapamycin (Fig. 4) and wortmannin (data not shown) still had strong inhibitory effect on p70$^{s6k}$ activation. These data indicate that in the absence of PKC activation,

**FIG. 2.** Effects of signal transduction pathway inhibitors on p70$^{s6k}$ activation. Cells were treated with either vehicle (0.1% Me$_2$SO or 0.001% ethanol; control) or a panel of inhibitors and cultured for 30 min, followed by FGF-2 stimulation. For FMA treatment, cells kept in the presence of 5 µM PMA for 24 h were stimulated with FGF-2. Cell lysates were immunoprecipitated using M5 antiserum, and $^{32}$P incorporation was measured. The plate was then exposed on an x-ray film. PIP, phosphatidylinositol phosphate.

**TABLE I**

| Treatment | cpm $^{32}$P detected in inositol phosphate peak corresponding to |
|-----------|------------------------------------------------------------------|
|           | PIP | PI(4,5)P$_2$ | PIP$_3$ |
| Control   | 437,949 | 814,501 | 263 |
| FGF-2, 10 min | 388,357 | 715,245 | 183 |
| FGF-2, 60 min | 940,102 | 1,250,156 | 431 |
| Control, 10 min | 846,112 | 1,030,819 | 259 |

* Picomoles of DAG/10$^6$ cells.

**TABLE II**

| Treatment | Wild-type receptor | Y766F receptor |
|-----------|--------------------|----------------|
| 0 time | 124 | 102 |
| Control, 10 min | 96 ± 18 | 105 ± 2 |
| FGF-2, 10 min | 207 ± 28 | 99 ± 4 |
| Control, 60 min | 127 ± 42 | 125 ± 5 |
| FGF-2, 60 min | 221 ± 28 | 131 ± 10 |

As shown in Fig. 1B, the extent of p70$^{s6k}$ activity at the mitogenically relevant, sustained phase was very similar in wild-type FGF receptor-1 and Y766F mutant receptor expressing cells. Moreover, Fig. 4 shows that the level of p70$^{s6k}$ activity in the Y766F mutant receptor expressing cells was not affected by treatment with bisindolylmaleimide, to inhibit PKC, or chronic exposure to PMA, to deplete cellular PKC levels. In these cells, rapamycin (Fig. 4) and wortmannin (data not shown) still had strong inhibitory effect on p70$^{s6k}$ activation. These data indicate that in the absence of PKC activation,
rapamycin- and wortmannin-sensitive pathways were modulated to compensate for the absence of the PKC-dependent pathway for p70<sub>65k</sub> activation.

**Role of p70<sub>65k</sub> in Biological Responses**—It has been reported that p70<sub>65k</sub> is involved in G₁-S transition in the cell cycle (11, 20) and therefore crucial for cell proliferation. We examined the effect of inhibiting FGF-2-stimulated p70<sub>65k</sub> in a murine brain capillary endothelial cell line undergoing proliferation or differentiation. The cell line was established from H-2K<sub>b</sub>-tsA58 SV40 large T transgenic mice (26). We have shown that, dependent on the growth conditions, the cells will either differentiate (i.e. form tube-like structures when grown on a collagen-coated dish or in three-dimensional collagen gels) or proliferate (when grown on fibronectin-coated dishes) in response to FGF-2. Cells cultured on fibronectin-coated dishes never showed a differentiated phenotype. On the other hand, cells grown on a collagen-coated dishes failed to grow in response to FGF-2 treatment as judged from lack of increase in labeling index.

We analyzed the parental and chimeric receptor expressing endothelial cells for their level of activated p70<sub>65k</sub> in response to FGF-2 treatment. As seen in Fig. 5, prominent activation of p70<sub>65k</sub> was seen in cells grown on fibronectin. A considerably weaker activation of p70<sub>65k</sub> was seen in cells grown on collagen gels (in cells stimulated for 30 min the extent of p70 activation was less than 30% of that demonstrated in cells cultured on fibronectin).

Next, we treated the endothelial cells, grown on fibronectin or in collagen gels, with rapamycin to block p70<sub>65k</sub> activation in response to FGF-2. The effects of rapamycin on proliferation was analyzed by assessing the increase in cell number of cells in fibronectin-coated wells. As seen in Fig. 6, rapamycin treatment inhibited proliferation of cells treated with FGF-2 in 0.2% serum (data not shown) or 2% serum (Fig. 6) in a dose-dependent manner. However, tube formation of endothelial cells in collagen gels in response to FGF-2 was not affected even by a high dose of rapamycin (Fig. 7), in agreement with the inefficient p70<sub>65k</sub> activation, in these cultures.

**DISCUSSION**

Here we show that FGF receptor-1 mediates activation of p70<sub>65k</sub> in different cell types and that treatment of the cells with different drugs, including rapamycin, wortmannin, and bisindolylmaleimide, inhibits p70<sub>65k</sub> activation. These drugs are known to attenuate signal transduction pathways involved in regulation of the serine/threonine kinase. However, the relationship between these pathways remains unclear. The immunosuppressant drug rapamycin forms a complex with FKBP-12, a peptidyl-propyl cis-trans-isomerase that may be involved in protein folding, and the complex binds and inhibits the function of yeast and mammalian TOR (9). The TOR proteins, which recently have been identified, all have a structural domain similar to PI3-kinase and PI4-kinase; however, phylogenetic analysis shows that the TOR proteins constitute a distinct family of lipid kinases (16). According to recent reports, mammalian TOR, derived from rat brain, possesses PI4-kinase activity (34, 35). Whether this activity is intrinsic to TOR has been disputed, based on the finding that kinase-inactive TOR possessed intact level of lipid kinase activity, as compared with wild-type TOR (18). In addition, the PI-4 kinase activity was not affected by rapamycin (see review, Ref. 36). In any case, TOR appears to be obligatory for S6 kinase activation, since rapamycin inhibits S6 kinase activation independent of cell

---

<sup>2</sup>S. Kanda, B. Tomasini-Johansson, P. Klint, K. Rubin, and L. Claesson-Welsh, submitted for publication.
type and stimulus (11, 12). The rapamycin-FKBP-12 complex does not appear to inhibit the kinase activity of TOR proteins, at least in yeast. Instead, inhibition might be exerted on the level of TOR protein binding to or phosphorylation of \( G_1 \) effectors (16). How TOR couples to upstream elements, such as receptor tyrosine kinases, has not been elucidated. It also remains to be shown whether TOR directly regulates S6 kinase activity. Indeed, recent data show that an amino-terminally truncated mutant of S6 kinase, which no longer is inhibited by rapamycin, still becomes phosphorylated at the critical regulatory Thr-389 site in response to serum, indicating that TOR is not involved in this phosphorylation (37).

The level of activation of \( p70^{66K} \) by FGF was about 3-fold as compared with basal, in different cell types; this is about half of the level of activation of \( p70^{66K} \) seen in PDGF-stimulated cells (data not shown). This difference in efficiency between FGF- and PDGF-stimulated \( p70^{66K} \) activation might be due to PDGF initiating multiple pathways leading to \( p70^{66K} \) activation. Thus, previous studies using the fungal metabolite wortmannin, to study the regulation of \( p70^{66K} \) activation in PDGF-stimulated cells, has implicated PI3-kinase as an upstream regulator of \( p70^{66K} \) (38). In agreement, expression of constitutively active PI3-kinase resulted in substantial activation of p70 (39). Rapamycin and wortmannin inhibit phosphorylation at the same set of serine sites in p70\(^{66K}\), indicating that these drugs target the same pathway (15). However, since susceptibility to wortmannin and rapamycin has been mapped to different domains of \( p70^{66K} \) (40) the input signals that these drugs modulate appear to be different. The serine/threonine kinase c-Akt/protein kinase B has been postulated to bridge between PI3-kinase and \( p70^{66K} \), possibly with several intermediates (41, 42). The ability of wortmannin to inhibit \( p70^{66K} \) kinase activity independent of PI3-kinase was also shown by Hara et al. (43). In cells expressing \( \Delta p85 \), a dominant negative form of the regulatory subunit of PI3-kinase, S6 kinase was still activated in response to insulin. In contrast, wortmannin treatment attenuated insulin-induced S6 kinase activity; \( p70^{66K} \) itself appears not to be inhibited by wortmannin (35); different reports show lack of effect on TOR/FKBP-12-rapamycin-associated protein (18) or inhibition of TOR autokinase activity (44) by wortmannin. Wortmannin inhibits PI3-kinase at m concentrations (45) but also affects other enzymes, such as phospholipase \( A_2 \) (30). The PI3-kinase inhibitor Ly294002 also efficiently inhibits S6 kinase activation in FGF-2-stimulated L6 cells expressing FGFR-1 (data not shown). Although LY294002 has been suggested to be a more specific PI3-kinase inhibitor, the range of targets for this drug as well as wortmannin is still not clear (46, 47). Thus, it is possible that the true target of wortmannin in the S6 kinase pathway is not PI3-kinase but a so far unidentified lipid kinase, or another type of enzyme, upstream of S6 kinase.

PKC is activated in response to FGF receptor-1 stimulation through PLC-\( \gamma \)-catalyzed hydrolysis of PIP\(_2\) to DAG and phosphatidylinositol 3,4,5-trisphosphate (48). DAG is also formed as a result of hydrolysis of phosphatidylycerol; the kinetics of phosphatidylerol-generated DAG formation are different, with a later and sustained peak than for PIP\(_2\)-derived DAG. One of the enzymes responsible for generation of phosphatidlycerol-activated DAG is phospholipase \( D \), which is activated by FGF-2. In cells expressing an FGF receptor-1 mutant lacking the major autophosphorylation site, Tyr-766, neither PLC-\( \gamma \) (49, 50) nor phospholipase \( D \) is activated in response to FGF-2. In consequence, DAG formation and PKC activation are suppressed. S6 kinase \( p70 \) activation in FGF-2-stimulated L6 cells expressing the Y766F mutant was only moderately affected (Fig. 1B). Treatment of cells expressing either the wild-type or mutant receptors with bisindolylmaleimide or PMA inhibited \( p70^{66K} \) in the wild-type receptor cells but had no effect in cells expressing the mutant receptor. We infer from these data that other pathways, such as the rapamycin- and wortmannin-sensitive pathways, for \( p70^{66K} \) activation were up-regulated in response to FGF-2 stimulation of the Y766F mutant, as compared with the wild-type FGFR-1. It has been shown that \( p70^{66K} \) activity is induced in response to insulin, even though PKC is not activated by this mitogen. Furthermore, inhibition of PKC attenuates EGF-induced but not PDGF-induced \( p70^{66K} \) activation (51). Thus, PKC appears to play a modulatory role in \( p70^{66K} \) activation but is not absolutely required.

The \( p70^{66K} \)-mediated phosphorylation of the 40 S ribosomal subunit S6 has been implicated in the selective up-regulation of a family of essential gene products. Inhibition of S6 phosphorylation, using neutralizing antibodies, or treatment with rapamycin, leads to a block in cell cycle progression (2, 9). However, although rapamycin blocks \( p70^{66K} \) activation in most cell types, it impedes cell cycle progression preferentially in hematopoietic cells (11). This has been suggested to be due to the redundancy of signal transduction pathways involved in the proliferative response. FGF-induced proliferation of an endothelial cell line was efficiently blocked by rapamycin. Dependent on the culture conditions, these cells form tubes in response to FGF-2 treatment. This response was not affected by rapamycin, which is in agreement that tube formation is independent of DNA synthesis.\(^4\) A critical task for the future is to identify the point in G\(_1\) progression, which is targeted by S6 kinase.

Acknowledgments—We thank Dr. Stefan Wennstrom for advice on PI3-kinase assay and lnggard Schiller for secretarial assistance.

REFERENCES

1. Jenö, P., Ballou, L. M., Novak-Hefer, I., and Thomas, G. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 406–410
2. Proud, C. G. (1996) Trends Biochem. Sci. 21, 181–185

---

\(^3\) M. Cross and M. Wakelam, manuscript in preparation.

\(^4\) S. Kanda, unpublished observations.
3. Reinhard, C., Thomas, G., and Koza, S. C. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 4052–4056
4. Groe, J. R., Banerjee, P., Balasubramanyam, A., Coffer, P. J., Price, D. J., Arru, J., and Woodgett, J. R. (1991) Mol. Cell. Biol. 11, 5541–5550
5. Ferrn, S., Bannwarth, W., Morley, S. J., Totty, N. F., and Thomas, G. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 7282–7286
6. Mukhopadhyay, N. K., Price, D. J., Kryakis, J. M., Pelech, S., Sanghera, J., and Arru, J. (1992) J. Biol. Chem. 267, 3325–3335
7. Ballou, L. M., Lurther, H., and Thomas, G. (1991) Nature 349, 348–350
8. Ming, X. P., Burgering, B. M. T., Wennstrom, S., Claesson-Welsh, L., Heldin, C. H., Bos, J. L., Kozma, S. C., and Thomas, G. (1994) Nature 371, 426–429
9. Chou, M. M., and Blenis, J. (1995) Curr. Opin. Cell Biol. 7, 806–814
10. Chou, M. M., and Blenis, J. (1996) Cell 85, 573–583
11. Kuo, C. J., Chung, J., Fiorentino, D. F., Flanagan, W. M., and Blenis, J. (1992) J. Biol. Chem. 267, 26068–26075
12. Chung, J., Kuo, C. J., Crabtree, G. R., and Blenis, J. (1992) Cell 70, 69–83
13. Price, D. J., Grove, J. R., Calvo, Y., Arru, J., and Bierer, B. E. (1992) Science 257, 973–977
14. Grove, J. R., Banerjee, P., Balasubramanyam, A., Coffer, P. J., Price, D. J., Arru, J., and Woodgett, J. R. (1991) Mol. Cell. Biol. 11, 919–925
15. Han, J.-W., Pearson, R. B., Dennis, P. B., and Thomas, G. (1995) J. Biol. Chem. 270, 21396–21403
16. Zheng, X.-F., Fiorentino, D., Chen, J., Crabtree, G. R., and Schreiber, S. L. (1995) Cell 82, 121–130
17. Brown, E. J., Albers, M. W., Shin, T. B., Ichikawa, K., Keith, C. T., Lane, W. S., and Schreiber, S. L. (1994) Nature 369, 756–758
18. Brown, E. J., Beal, P. A., Keith, C. T., Chen, J. B., and Schreiber, S. L. (1995) Nature 377, 441–446
19. Pearson, R. B., Dennis, P. B., Han, J.-W., Williamson, N. A., Kozma, S. C., Wettenthal, R. E. H., and Thomas, G. (1995) EMBO J. 14, 5279–5287
20. Lane, H. A., Fernandez, A., Lamb, N. C. J., and Thomas, G. (1990) Nature 363, 170–172
21. Reinhard, C., Fernandez, A., Lamb, N. C. J., and Thomas, G. (1994) EMBO J. 13, 1557–1565
22. Friesel, R. E., and Macey, T. (1995) FASEB J. 9, 919–925
23. Partanen, J., Vainikka, S., Korhonen, J., Armstrong, E., and Ahlatalo, K. (1992) Prog. Growth Factor Res. 4, 69–83
24. Giro, D., and Yarden, Y. (1992) FASEB J. 6, 3362–3369
25. Mohammadi, M., Honegger, A. M., Rotin, D., Fischer, R., Bellot, F., Li, W., Dinnel, C. A., Jaye, M., Rubinstein, M., and Schlessinger, J. (1991) Mol. Cell. Biol. 11, 5068–5078
26. Kanda, S., Landgren, E., Ljungstrom, M., and Claesson-Welsh, L. (1996) Cell Growth Differ. 7, 383–395
27. Kahn, C., Seuwen, K., Meloche, S., and Pouysegu, J. (1992) J. Biol. Chem. 267, 15305–15315
28. Klint, P., Kanda, S., and Claesson-Welsh, L. (1995) J. Biol. Chem. 270, 23357–23344
29. Wennstrom, S., Sandstrom, C., and Claesson-Welsh, L. (1991) Growth Factors 4, 197–208
30. Cross, M. J., Stewart, A., Hodgkin, M. N., Kerr, D. J., and Wakeland, M. J. O. (1995) J. Biol. Chem. 270, 25352–25355
31. Kanda, S., Sandstrom, C., and Claesson-Welsh, L. (1991) Growth Factors 4, 197–208
32. Chung, J., Kuo, C. J., Crabtree, G. R., and Blenis, J. (1992) Cell 70, 69–83
33. Suzuki, M., Olivier, A. R., Fabbro, D., and Thomas, G. (1989) Cell 57, 817–824
34. Wennstrom, S., Siegbahn, A., Yokote, K., Arvidsson, A.-K., Heldin, C.-H., Mori, S., and Claesson-Welsh, L. (1994) Oncogene 9, 651–660
35. Sabatini, D. M., Pierchala, B. A., Barrow, R. K., Schell, M. J., and Snyder, S. H. (1995) J. Biol. Chem. 270, 20875–20878
36. Cardenas, M. E., and Heitman, J. (1995) EMBO J. 14, 5892–5907
37. Hunter, T. (1995) Cell 83, 1–4
38. Dennis, P. B., Pulen, N., Koza, S. C., and Thomas, G. (1996) Mol. Cell. Biol. 16, 6242–6251
39. Chung, J., Grammer, T. C., Lemon, K. P., Kazlauskas, A., and Blenis, J. (1994) Nature 370, 71–75
40. Weng, Q.-P., Andrab, K., Krippel, A., Koza, M. T., Williams, L. T., and Arru, J. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 5744–5748
41. Weng, Q.-P., Andrab, K., Koza, M. T., Grove, J. R., and Arru, J. (1995) Mol. Cell. Biol. 15, 2333–2340
42. Burgering, B. M. T., and Coffer, P. J. (1995) Nature 376, 599–602
43. Franke, T. F., Yang, S.-I., Chan, T. O., Dutta, K., Kazlauskas, A., Morrison, D. K., Kaplan, D. R., and Tschil, P. N. (1995) Cell 84, 727–736
44. Brunn, G. J., Williams, J., Sabers, C., Wiederrecht, G., Lawrence, J. C., and Abraham, R. T. (1996) EMBO J. 15, 5256–5267
45. Yano, H., Nakashishi, S., Kimura, H., Hanai, N., Saitoh, Y., Fuku, Y., Hasumura, Y., and Matsuda, Y. (1993) J. Biol. Chem. 268, 25846–25856
46. Scheid, M. P., and Duronio, V. (1996) J. Biol. Chem. 271, 18134–18139
47. Hawes, B. E., Luttrell, L. M., van Biesen, T., and Lefkowitz, R. J. (1996) J. Biol. Chem. 271, 12333–12336
48. Berridge, M. J. (1993) Nature 361, 315–325
49. Peters, K. G., Marie, J., Wilson, E., Ives, H. E., Escobedo, J., Del Rosario, M., Mirda, D., and Williams, L. T. (1992) Nature 358, 678–681
50. Mohammadi, M., Dinnel, C. A., Li, W., Li, N., Spivak, T., Honegger, A. M., Jaye, M., and Schlessinger, J. (1992) Nature 358, 681–684
51. Susa, M., Vulevic, D., Lane, H. A., and Thomas, G. (1992) J. Biol. Chem. 267, 6905–6909
52. Han, J.-W., Pearson, R. B., Dennis, P. B., and Thomas, G. (1995) J. Biol. Chem. 270, 26068–26075