Signal Pathways Involved in Activation of p70\(_{S6K}^\) and Phosphorylation of 4E-BP1 following Exposure of Multiple Myeloma Tumor Cells to Interleukin-6*  

Yijiang Shi, Jung-hsin Hsu, Liping Hu, Joseph Gera, and Alan Lichtenstein†  

From the Department of Medicine, West Los Angeles Veterans Affairs-UCLA Medical Center and the Jonsson Comprehensive Cancer Center, Los Angeles, California 90073

Interleukin-6 (IL-6) is a prominent tumor growth factor for malignant multiple myeloma cells. In addition to its known activation of the Janus tyrosine kinase-STAT and RAS-MEK-ERK pathways, recent work suggests that IL-6 can also activate the phosphatidylinositol 3-kinase (PI3-K)/AKT kinase pathway in myeloma cells. Because activation of the PI3-K/AKT as well as RAS-MEK-ERK pathways may result in downstream stimulation of the p70\(_{S6K}^\) (p70) and phosphorylation of the 4E-BP1 translational repressor, we assessed these potential molecular targets in IL-6-treated myeloma cells. IL-6 rapidly activated p70 kinase activity and p70 phosphorylation. Activation was inhibited by wortmannin, rapamycin, and the ERK inhibitors PD98059 and UO126, as well as by a dominant negative mutant of AKT. The concurrent requirements for both ERK and PI3-K/AKT appeared to be a result of their ability to phosphorylate p70 on different residues. In contrast, IL-6-induced phosphorylation of 4E-BP1 was inhibited by rapamycin, wortmannin, and dominant negative AKT but ERK inhibitors had no effect, indicating ERK function was dispensable. In keeping with these data, a dominant active AKT mutant was sufficient to induce 4E-BP1 phosphorylation but could not by itself activate p70 kinase activity. Prevention of IL-6-induced p70 activation and 4E-BP1 phosphorylation by the mammalian target of rapamycin inhibitors rapamycin and CCI-779 resulted in inhibition of IL-6-induced myeloma cell growth. These results indicate that both ERK and PI3-K/AKT pathways are required for optimal IL-6-induced p70 activity, but PI3-K/AKT is sufficient for 4E-BP1 phosphorylation. Both effects are mediated via mammalian target of rapamycin function, and, furthermore, these effects are critical for IL-6-induced tumor cell growth.

* This work was supported by grants from the Department of Veterans Affairs including the Research Enhancement Awards Program entitled “Cancer Gene Medicine” and by a Year 2000 senior research award from the Multiple Myeloma Research Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
† To whom all correspondence should be addressed. Tel.: 310-268-3622; Fax: 310-268-4508; E-mail: alichten@ucla.edu.

‡ The abbreviations used are: IL-6, interleukin-6; IGF-1, insulin growth factor-1; p70\(_{S6K}^\), p70/95 ribosomal S6 kinase; PDK1, 3-phosphoinositide-dependent protein kinase-1; ERK, extracellular signal-regulated kinase; mTOR, mammalian target of rapamycin; PI3-K, phosphatidylinositol 3-kinase; MM, multiple myeloma; EGFP, enhanced green fluorescent protein; PH, pleckstrin homology; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; HA, hemagglutinin; m.o.i., multiplicity of infection.
tion of proliferation is thought to be uniquely the result of downstream signaling through the mammalian target of rapamycin (mTOR), which results in phosphorylation and activation of the p70^S6K (p70) and phosphorylation of the 4E-BP1 transactivation repressor. p70 activation results in increased phosphorylation of the 40 S ribosomal S6 protein and 4E-BP1 phosphorylation disrupts its interaction with the eIF-4E initiation factor, allowing eIF-4E to participate in assembly of a translation initiation complex. These events lead to translational up-regulation of the proteins needed for cell cycle transit. Our prior results indicated that the PI3-K/AKT pathway was more important in MM cell proliferative rather than survival responses (18). Thus, in the current study, we focused on its possible downstream activation of p70 and phosphorylation of 4E-BP1 in IL-6-treated MM cells. Because the RAS/RAF/MEK/ERK pathway can also mediate p70 (19) or 4E-BP1 phosphorylation (20), and is potentially activated by IL-6 in MM cells, we also tested the role of this cascade in p70/4E-BP1 phosphorylation.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Reagents—**The MM cell lines AF-10 and 8226 were kind gifts from Drs. J. Epstein (University of Arkansas, Little Rock, AR) and James Berenson (UCLA, Los Angeles, CA). The lines were maintained as previously described (2, 12). Recombinant IL-6 and insulin-like growth factor-1 (IGF-1) were purchased from R&D Systems (Minneapolis, MN). Phosphospecific antibodies were purchased from New England Biolabs. All other reagents were purchased from Sigma unless otherwise described.

**Expression Constructs—**The N terminus AKT construct HA-AKT (PH), which includes the PH domain but lacks the kinase domain and the HA-E40K construct, which contains a point mutation in its PH domain, have been previously described (21, 22). A recombinant adenovirus that encodes HA-AKT(PH) or HA-E40K was generated by homologous recombination in bacteria. The HA-tagged constructs were first cloned into pAdTrack-CMV, which also contains the green fluorescent protein gene. The resultant construct was linearized and transformed with the supercoiled adenoviral vector, pΔEasy-1 into Echerichio coli and recombinants selected in kanamycin and screened by restriction endonuclease digestion. The recombinant adenovirus was then transfected into 293 cells. Control pAdTrack-CMV recombinant virus was generated in identical fashion but without transgene insertion.

**Transfection—**Myeloma cells were transduced with adenovirus at varying m.o.i. values for 2 h. Virus was then washed away, and cells were resuspended in media with low serum concentration (1%) to minimize proliferation. At 24 and 48 h, enhanced green fluorescent protein (EGFP) fluorescence demonstrated that >85% of cells were successfully transduced when using m.o.i. values between 10 and 100.

**AKT Kinase Assay—**The AKT in vitro kinase assay utilized a non-radioactive kit purchased from New England Biolabs. AKT was immuno-nprecipitated from cell extracts and incubated with GST-3 fusion protein in the presence of ATP and kinase buffer. AKT-dependent GST-3 phosphorylation was detected by immunoblotting with a phosphospecific GST-3 antibody.

**p70 in Vitro Kinase Assay—**As previously described (23), p70^S6K was immunoprecipitated from myeloma cells with C18 antibody (Santa Cruz). p70 was then mixed with S6 substrate peptide (Upstate Biotechnology, Inc.) in kinase buffer in the presence of 200 μCi/ml [γ-^32P]ATP. The reaction was stopped by addition of 20 μl of 250 mM EDTA, followed by boiling for 5 min. The reaction mixture was then transferred to phosphocellulose columns (Pierce), which were centrifuged to separate free ^32P from S6-labeled ^3P. The amount of labeled S6 peptide adhered to the columns was then assayed by liquid scintillation.

**4E-BP-1 Phosphorylation Assay—**Phosphorylation of 4E-BP1 was detected by differential migration in SDS-PAGE. As previously described (24), protein lysates were first boiled and then incubated on ice. After centrifugation, supernatant protein was precipitated with 15% trichloroacetic acid for 1 h, followed by two washes with diethyl ether. The pellet was dissolved in Laemmli buffer and electrophoresed in 15% SDS-polyacrylamide gel. Membranes were blotted with anti-4E-BP1 antibody from Santa Cruz Chemicals.

**Cell Growth Assay—**MM cells were cultured at 2 × 10^6^ cells/ml in 6- or 12-well plates. Some groups were pretreated with rapamycin or CCI-779 for 2 h before adding IL-6 at 100 units/ml. After 48 h, cells were harvested and viable recovery determined by trypan blue staining.

**RESULTS**

**IL-6 Activates mTOR-dependent p70^S6K Activity in Myeloma Cells**

To assess the activation and roles of p70/4E-BP1 phosphorylation in IL-6-stimulated MM cells, we primarily used the AF-10 MM cell line for these studies for the following reasons. 1) Although the line can grow in the absence of IL-6, addition of exogenous IL-6 reproducibly increases proliferation; 2) the two dominant IL-6 signal pathways that are classically stimulated in MM cells, STAT1/STAT3 activation and RAS/RAF/MEK/ERK activation, are consistently activated in AF-10 cells; and 3) IL-6 rapidly activates PI3-K and AKT in these cells. In addition, AF-10 cells have functional IGF-1 receptors, allowing us to use IGF-1 stimulation as a positive control for p70/4E-BP1 phosphorylation.

Using a p70 in vitro kinase assay, we could demonstrate that IL-6 rapidly activated p70 kinase function (Fig. 1A) with kinetics and a magnitude that was similar to that achieved with IGF-1. IL-6-stimulated activity developed between 5 and 15 min of incubation and peaked at 15 min (2.5-fold over unstimulated control cells). Activity slightly decreased by 30 min (2.1-fold over control) and returned to base line by 60 min (1.2-fold of control; data not shown). The results shown in Fig. 1 were obtained from cells stimulated with 100 units/ml IL-6. This was
optimal IL-6-induced stimulation, as experiments using 1000 units/ml did not result in greater kinase stimulation, whereas 1 and 10 units/ml were less effective (data not shown). IGF-1 likewise stimulated kinase activity in AF-10 cells. Pretreatment with the mTOR inhibitor rapamycin completely abrogated the ability of both IL-6 and IGF-1 to activate kinase activity (Fig. 1A).

IL-6-induced p70 kinase activity correlated with IL-6-induced p70 phosphorylation. Utilizing phosphospecific p70 antibodies and IGF-1 treatment as a positive control, we could show that IL-6 induced phosphorylation at serine 411, threonine 421/serine 424, and threonine 389 (Fig. 1B). These are critical residues, phosphorylation of which results in optimal p70 kinase activity. Clear induction of phosphorylation was seen by 15 min of incubation. As shown, pretreatment with rapamycin prevented phosphorylation of all these residues. Thus, as for p70 kinase activity, mTOR regulates IL-6-dependent p70 phosphorylation.

**IL-6-induced p70s6k Activity Is Dependent upon Both PI3-K/AKT and MEK/ERK Pathways**

IL-6 is capable of activating the PI3-K/AKT pathway (12) as well as the RAS/RAF/MEK/ERK (8, 9) pathway in MM cells. To specifically test activation of these pathways in the AF-10 MM cell line, we exposed cells to IL-6 (100 units/ml) for varying durations and performed Western blot for the phosphorylated, activated form of AKT or p42/p44 ERK. In five separate experiments, exposure to IL-6 consistently induced phosphorylation of ERK and AKT in AF-10 cells. In Fig. 2A, results of two of these experiments are shown. In the left panel, p42 ERK demonstrates an increase in phosphorylation by 5 min, which increases to maximal levels by 30 min, and p44 ERK also demonstrates maximal phosphorylation by 30 min. In the second experiment shown (right panel), maximal phosphorylation is again demonstrated by 30 min of IL-6 exposure, although the major effect (in this experiment) was on p44 ERK (3.9-fold increase by densitometry) as compared with p42 (only 2.1-fold increase). Similarly, IL-6 markedly induced AKT phosphorylation at 15 and 30 min (one representative experiment shown in Fig. 2B).

In these experiments, we also tested the effectiveness and specificity of wortmannin, a PI3-K inhibitor, and U0126 and PD98059, two unrelated MEK/ERK inhibitors. Preliminary experiments identified the range of concentrations that effectively inhibit either AKT or ERK phosphorylation. We then tested effective lower concentrations of the inhibitors to test the specificity of inhibition. At 0.1 μM, wortmannin (W) completely abrogated IL-6-dependent AKT phosphorylation (Fig. 2A, right panel) but had no effect on ERK phosphorylation (Fig. 2A, right panel). In contrast, U0126 (UO) at 12.5 μM, and PD98059 (PD) at 50 μM, completely inhibited IL-6-dependent ERK phosphorylation (Fig. 2A, left panel) but had no effect on AKT phosphorylation (Fig. 2B).

We next used these inhibitors at the same effective and specific concentrations to test effects on p70s6 kinase activity. p70 in vitro kinase activity in AF-10 cells stimulated by IL-6 was increased 2.4-fold (mean of four experiments) over control nonstimulated cells in this set of experiments (Fig. 3A). In the presence of wortmannin (W), U0126 (UO), and PD98059 (PD), the IL-6-induced increase was significantly curtailed (only 1.2-, 1.35-, and 1.4-fold increase over control non-IL-6-stimulated cells, respectively; p < 0.05; Fig. 3A).

These data confirmed involvement of PI3-K and MEK/ERK in p70 activation. To test involvement of AKT, we expressed a dominant negative AKT construct in AF-10 cells by adenoviral transfection. Our adenoviral vector expresses EGFP, which allowed us to test transfection efficiency in AF-10 cells. Fluorescent microscopy demonstrated very effective transfection efficiency at low m.o.i. (>85% at m.o.i. 10). The dominant negative HA-tagged AKT construct, termed PH (21), was trans-
24 h later, stimulated with or without IL-6 for 15 min. PH and, that pretreatment was with or without PD98059 or UO126. In C, cells were transfected with control or dominant negative PH AKT (PH) and, 24 h later, stimulated with or without IL-6 for 15 min.

affected into AF-10 cells, and anti-HA immunoblots as well as AKT in vitro kinase assays in IL-6-treated cells demonstrated expression of the truncated PH construct (Fig. 3B) and inhibition of IL-6-induced activation of endogenous AKT (Fig. 3C). p70 in vitro kinase assays also demonstrated a significant inhibition (p < 0.05) of IL-6-induced p70 kinase activity in PH-transfected cells (Fig. 3A). As shown, EGFP control-transfected cells still were capable of increasing their p70 activity to >2-fold increase over non-IL-6-treated cells, whereas dominant negative PH-transfected AF-10 cells only could increase activity to 1.2-fold over control when exposed to IL-6. Thus, the PI3-K/AKT pathway and the MEK/ERK pathway both regulate IL-6-dependent p70 kinase activity. As inhibition of each distinct pathway alone could abrogate p70 activity, the results suggest that the two pathways are additive or synergistic rather than overlapping.

Differential Phosphorylation of Specific p70 Residues in IL-6-stimulated MM Cells

Phosphorylation of p70 on Thr^{421}/Ser^{424}—The p70 kinase is activated by hierarchical phosphorylation at multiple sites (25). Initial phosphorylation at serine 411, threonine 421, and serine 424 in the auto-inhibitory domain relieves pseudosubstrate suppression. Activation also requires subsequent PI3-K-dependent phosphorylation at threonine 389 in the adjacent linker domain. These events then synergize to allow phosphorylation at threonine 229 in the catalytic domain. Phosphorylation at Thr^{229} then results in optimal kinase activity. Thus, one possible explanation for a concurrent requirement of MEK/ERK and PI3-K/AKT in p70 activation was that each pathway was necessary for differential phosphorylation steps on different residues of p70 to allow full activation. We, again, used the phosphospecific p70 antibodies to test this hypothesis. Initial p70 phosphorylation on Thr^{421}/Ser^{424} in the auto-inhibitory domain was activated by IL-6 after 15 and 30 min of incubation and returned toward base line at 60 min (Fig. 4, A and B). Wortmannin, used in concentrations that abrogated Akt activation, had no effect on Thr^{421}/Ser^{424} phosphorylation (Fig. 4A). In contrast, the MEK/ERK inhibitors PD98059 and UO126 both inhibited Thr^{421}/Ser^{424} phosphorylation induced by IL-6 (Fig. 4B). These data support the hypothesis that IL-6-induced phosphorylation of p70 on Thr^{421}/Ser^{424} is dependent upon signaling through RAS/RAF/MEK/ERK. Further evidence that the PI3-K/AKT pathway was not critical for IL-6-dependent Thr^{421}/Ser^{424} phosphorylation was obtained with use of the PH dominant negative-expressing adenovirus. As shown in Fig. 4C, there was no effect on IL-6-dependent Thr^{421}/Ser^{424} phosphorylation when AF-10 cells were transfected with the PH construct. Thus, although PI3-K/AKT function was critical for p70 kinase activity (Fig. 3) and, as will be shown below, for phosphorylation at other p70 residues, it was not required for Thr^{421}/Ser^{424} phosphorylation.

Phosphorylation of p70 on Serine 411—In contrast to the above results on phosphorylation of Thr^{421}/Ser^{424}, phosphorylation of Ser^{411} was regulated by both MEK/ERK and PI3-K/AKT pathways. As shown in Fig. 5A, IL-6-induced Ser^{411} phosphorylation was clearly inhibited by pretreatment with PD98059, UO126, and wortmannin. In addition, IL-6-treated, PH-transfected AF-10 cells were incapable of Ser^{411} phosphorylation compared with EGFP control (Fig. 5B). These data indicate independent regulation of Ser^{411} phosphorylation by both pathways.

Phosphorylation of p70 on Threonine 389—Similar results were obtained using the phosphospecific Thr^{389} anti-p70 antibody. Significant inhibition of IL-6-induced Thr^{389} phosphorylation was afforded by wortmannin, PD98059, and UO126 (Fig. 6A), as well as the PH dominant negative AKT construct (Fig. 6B).

Effect of Constitutively Expressed Activated AKT

Although the above experiments utilizing the PH AKT dominant negative confirm an AKT-dependent pathway for IL-6-induced p70 kinase activation and phosphorylation, the results also suggest an interaction between AKT and the MEK/ERK pathway is required for p70 activity. To further support the notion that AKT must interact with MEK/ERK-dependent events, and, by itself, is insufficient to activate p70, we transfected a dominant active form of AKT, E40K (22), which possesses enhanced kinase activity caused by a point mutation resulting in an increased affinity of the PH domain for second messenger phospholipids (22). AF-10 cells were transfected with the adenovirus expressing E40K control virus, and, 24 h later, cells were harvested and assayed. As with the PH dominant negative AKT-expressing adenovirus (above), the transfection efficiency was again very high (>85% at m.o.i. 10). The transfected AKT was constitutively phosphorylated as shown by immunoblot assay (Fig. 7B). In addition, the E40K was functional as a kinase inducing BAD phosphorylation and GSK-3 phosphorylation. However, MM cells transfected with...
ERK1 and ERK2 (26). Initial in vitro demonstrated heightened phosphorylation of the Ser411 p70 noblot analyses demonstrated that E40K-transfected cells only activation, activated AKT was not sufficient. Additional immu-AKT confirmed a requirement for AKT in IL-6-induced p70 A. Thus, although experiments with the dominant negative E40K at m.o.i. 10 or 100 did not demonstrate heightened p70 activation is induced (12). In addition, the 8226 MM line ex-modulated by IL-6 in 8226 cells, and, most importantly, AKT this cytokine (26). Several signal transduction pathways are 7 of Thr421/Ser424 or Thr389 induced by transfection of E40K. B. Phosphorylation of p70 on Thr389. FIG. 7. AF-10 cells transfected with either control (C) or E40K (E) active AKT construct (at m.o.i. 10 or 100). Twenty-four h later, cells were not treated or treated with IL-6 for 15 min. In A (left two columns), p70 was immunoprecipitated from control-transfected (C) or E40K-transfected (E) cells infected with m.o.i. = 10 and kinase assay performed. Middle two columns demonstrate p70 kinase activity from cells infected with m.o.i. = 100, and right two columns are cells infected with m.o.i. of 10 and then treated with IL-6 for 15 min (C/IL6 and E/IL6). Results are means ± S.D. of three separate experiments. In B, AF-10 cells were transfected with control (C) or E40K (E), and, 24 h later, immunoblot assay performed for phosphorylated AKT (AKT-P), total AKT, phosphorylated GSK-3 (GSK-3-P), phosphorylated BAD (BAD-P), phosphorylated p70 on different residues, and total p70. E40K at m.o.i. 10 or 100 did not demonstrate heightened p70 kinase activity, even when further stimulated with IL-6 (Fig. 7A). Thus, although experiments with the dominant negative AKT confirmed a requirement for AKT in IL-6-induced p70 activation, activated AKT was not sufficient. Additional immunoblot analyses demonstrated that E40K-transfected cells only demonstrated heightened phosphorylation of the Ser311 p70 residue (Fig. 7B, right panel). There was little phosphorylation of Thr421, Ser422, or Thr389 induced by transfection of E40K.

Studied on 8226 MM Cells

To demonstrate that the above results were not peculiar to AF-10 MM cells, we also studied a second MM cell line, 8226, in a limited number of experiments. The 8226 cell line expresses functional IL-6 receptors and is protected against apoptosis by this cytokine (26). Several signal transduction pathways are modulated by IL-6 in 8226 cells, and, most importantly, AKT activation is induced (12). In addition, the 8226 MM line expresses a mutated ras oncogene and constitutively activated ERK1 and ERK2 (26). Initial in vitro kinase assays demonstrated that IL-6 activated p70 kinase activity in 8226 cells by 15 and 30 min of incubation (Fig. 8A). At 15 min the increase was at 1.7-fold of control (untreated 8226 cells) and, at 30 min, it was 2.2-fold of control. Wortmannin (0.1 μM), PD98059 (50 μM), and rapamycin (100 nM) all significantly (p < 0.05) inhibited activation of the kinase in 8226 cells. As the transfection efficiency with adenoviral vectors was as high in 8226 cells (>90% at m.o.i. 10) as it was in AF-10 MM cells, we could test the effect of the dominant negative PH AKT. As shown in Fig. 8B, PH-transfected 8226 cells were incapable of activating p70 kinase activity when exposed to IL-6 (p < 0.05). These data are similar to the above results with the AF-10 MM cell line in demonstrating a requirement for both MEK/ERK function as well as PI3-K/AKT activity in IL-6 induction of p70 activity. A similar pattern of results was seen when phosphorylation of p70 on Thr389 was studied. As shown in Fig. 8C, wortmannin, rapamycin, and PD98059 all curtailed the ability of IL-6 to induce Thr389 phosphorylation on p70 in 8226 cells.

In Vitro Phosphorylation of p70

The above data in both AF-10 and 8226 MM cells indicated a requirement for the MEK/ERK pathway in p70 phosphorylation on Thr389, a key residue required for optimal induction of kinase activity. To determine whether ERK could directly phosphorylate this residue, we immunoprecipitated p70 from rest-IL-6 cells (cultured in the absence of serum) and incubated it in vitro with activated ERK1 or ERK2 (purchased from Upstate Biotechnology, Inc.) for 10 min at 30 °C. Phosphorylation of p70 was then assayed by immunoblot with phosphospecific antibodies. As shown in Fig. 9, activated ERK2 and, to a lesser extent, ERK1 were capable of in vitro phosphorylation of Ser411 and Thr422/Ser424 when added at 10 ng/μl reaction. When 10-fold less activated ERK1 and ERK2 was added, little substrate phosphorylation was seen (data not shown). In contrast, activated ERKs could not phosphorylate Thr389 on the immunoprecipitated p70 (Fig. 9). These data indicate ERK is incapable of directly phosphorylating Thr389 and suggest that the MEK/ERK requirements for Thr389 phosphorylation are the result of phosphorylation at the other residues, which allow access of Thr389 to different kinases.

IL-6 Induction of 4E-BP1 Phosphorylation

To investigate the second potential downstream target of the PI3-K/AKT/mTOR pathway, 4E-BP1, we used immunoblot assays that allowed discrimination of three forms of 4E-BP1, depending upon their state of phosphorylation. 4E-BP1 was resolved into as many as three separate bands, designated α, β, and γ in order of increasing electrophoretic mobility. These forms arise from differences in the phosphorylation state with an increased phosphorylation causing a decrease in mobility. Thus, β and γ represent the more phosphorylated forms of 4E-BP1. As shown in Fig. 10A, IL-6 treatment of AF-10 cells resulted in hyperphosphorylation of 4E-BP1, as shown by an increase in the relative proportion of the more highly phosphorylated γ and β forms of 4E-BP1 (left panel). The effect of IL-6 was equal or even more impressive than IGF in these experiments. In the presence of the MEK/ERK inhibitor, PD98059, used in the same concentration that inhibited p70 kinase activity (50 μM), the IL-6-induced hyperphosphorylation of 4E-BP1 was unaffected. However, both wortmannin and rapamycin prevented 4E-BP1 phosphorylation (Fig. 10A). As shown, there was minimal detection of the γ and β forms of 4E-BP1 in these inhibitor-treated cells. To test the role of AKT in IL-6-induced phosphorylation of 4E-BP1, we transfected AF-10 cells with the same control (con) and dominant negative PH AKT-expressing adenoviral vectors as described above. As shown in

![Fig. 6. Phosphorylation of p70 on Thr389](https://example.com/fig6.png)

**Fig. 6. Phosphorylation of p70 on Thr389**. In A, cells were pre-treated with wortmannin, PD98059, or U0126 and then stimulated with IL-6 for 0, 15, or 30 min. Thr389 phosphorylation was assayed by immunoblot. In B, cells were transfected with either control (EGFP) or PH constructs and, 24 h later, cells stimulated with IL-6 for 0, 15, or 30 min.
FIG. 8. Studies in 8226 myeloma cells. In A, 8226 cells were pretreated with wortmannin, PD98059, or rapamycin and then stimulated with or without IL-6 for 15 or 30 min. p70 kinase activity was then assayed and data expressed as fold increase over control (no IL-6), mean ± S.D. of three separate experiments. In B, 8226 cells were transfected with either control (C) or PH (PH) dominant negative AKT and, 24 h later, stimulated with or without IL-6 for 20 min. p70 kinase activity was then assessed. Results are mean ± S.D. of three experiments. In C (upper panel), 8226 cells were pretreated with or without (no inhib) rapamycin or PD98059 and then stimulated with or without IL-6 for 0, 15, or 30 min. p70 phosphorylation on Thr389 was then assessed by immunoblot. In C (lower panel), 8226 cells were first pretreated with or without (no inhib) wortmannin.
assayed for 4E-BP1 phosphorylation. Corresponding increase in the hyperphosphorylated forms of 4E-BP1 was resolved by migration in SDS-PAGE. In control-transfected cells exposed to IL-6, AF-10 cells transfected with either vector demonstrated little, if any, activation of p70 kinase activity or p70/4E-BP1 phosphorylation. In contrast, both drugs effectively abrogated the ability of IL-6 to stimulate MM cell growth in a concentration-dependent fashion.

**DISCUSSION**

The results of this study demonstrate that concurrent stimulation of the MEK/ERK and PI3-K/AKT cascades is required for activation of the p70 kinase in IL-6-treated myeloma cells. A similar interaction between the two upstream pathways was required for p70 phosphorylation on Thr^389, a key residue required for kinase activation. In contrast, although the P13-K/ AKT pathway was required for IL-6-dependent p70 phosphorylation, the MEK/ERK pathway was dispensable. Both IL-6-dependent p70 activation/phosphorylation and 4E-BP1 phosphorylation was inhibited by rapamycin, and this drug also inhibited IL-6-dependent cell growth.

Our results confirm and extend a previous study, which documented the ability of gp130-generated signals to activate the p70 kinase in leukemia inhibitory factor (LIF)-treated cardiac myocytes (28). LIF induced p70 activation, which was sensitive to wortmannin and rapamycin, implicating P13-K and mTOR. However, because wortmannin also inhibited LIF-dependent activation of mitogen-activated protein kinases in these cells, it was not clear whether ERK mitogen-activated protein kinases or other P13-K targets such as PDK1 or AKT were upstream activators of p70. In contrast, wortmannin has no effect on IL-6-dependent phosphorylation of ERKs in our myeloma cells. Thus, the ability of wortmannin to inhibit p70 activation/phosphorylation in myeloma cells was independent of any effects on ERK. In addition, use of a dominant negative construct confirmed a role for AKT downstream of PI3-K in IL-6-dependent p70 activation. However, two unrelated MEK/
ERK inhibitors, which had no effect on AKT activation, also significantly curtailed IL-6-induced p70 activation. In addition, a constitutively active AKT construct could not, by itself, induce p70 activation. Taken together, these results support independent requirements for both MEK/ERK and PI3-K/AKT pathways in IL-6-induced p70 kinase activation. Concurrent requirements for these two pathways in p70 activation have been previously shown in insulin-stimulated (19) and UV-stimulated (30) cells.

Because the p70 kinase is activated by phosphorylation at several residues, we considered the possibility that the two required upstream activating pathways were responsible for separate phosphorylation events. In keeping with this hypothesis, MEK/ERK inhibitors effectively prevented IL-6-induced p70 phosphorylation at Thr\textsuperscript{422}/Ser\textsuperscript{424}, whereas wortmannin and the dominant negative AKT had no effect. These data are consistent with prior studies that identified these residues as targets for proline-directed ERK kinases (31). As Thr\textsuperscript{422}/Ser\textsuperscript{424} phosphorylation in the p70 auto-inhibitory domain is required to relieve pseudosubstrate inhibition and allow subsequent phosphorylation at Thr\textsuperscript{389}, this event could explain the requirement for MEK/ERK in optimal p70 activation and Thr\textsuperscript{389} phosphorylation. The inability of activated ERK1 or ERK2 to directly phosphorylate Thr\textsuperscript{389} in vitro further supports this notion. On the other hand, wortmannin and the dominant negative AKT clearly inhibited Thr\textsuperscript{389} phosphorylation, which is consistent with prior studies that indicate a PI3-K-dependent pathway is crucial for Thr\textsuperscript{389} phosphorylation.

The results of p70 Ser\textsuperscript{411} phosphorylation are not as easy to explain. This residue is also in the C-terminal auto-inhibitory domain, and its phosphorylation would also be important for relieving pseudosubstrate inhibition. Our results suggest both MEK/ERK or PI3-K/AKT pathways may induce Ser\textsuperscript{411} phosphorylation. A previous study (30) demonstrated that UV-induced Ser\textsuperscript{411} phosphorylation was inhibited by a MEK/ERK inhibitor and dominant negative ERK and Jun kinase constructs. As Jun kinase activity is actually decreased by IL-6 treatment of myeloma cells (26), it is unlikely to be mediating Ser\textsuperscript{411} phosphorylation in our cells.

The dual requirement for MEK/ERK and PI3-K/AKT pathways in IL-6 induction of p70 kinase activity and Thr\textsuperscript{389} phosphorylation was also demonstrated in a second myeloma cell model, the 8226 line. This is of particular interest in that the MEK/ERK pathway is constitutively activated in that line because of an oncogenic \textit{ras} mutation and IL-6 is incapable of further ERK activation (26). The ability of the MEK/ERK inhibitor PD98059 to prevent IL-6-induced p70 kinase activity and Thr\textsuperscript{389} phosphorylation in 8226 cells suggests some MEK/ERK-mediated p70 phosphorylation must be present, either constitutively or cytokine-induced, to allow for further p70 kinase activation. Because IL-6 does not induce proliferation in 8226 cells, it is also clear that p70 activation, by itself, is not sufficient for a complete proliferative response.

The role of AKT in activation of p70 is controversial. Although some membrane-localized constitutively active forms of AKT can induce p70 activation (32, 33), similarly active but non-membrane-localized mutants may not (33). Those results suggest that an additional membrane-localized, p70-activating kinase might be stimulated by the co-localized AKT. In addition, some dominant-interfering AKT constructs significantly inhibit p70 activation (34), whereas others have little effect (33). In contrast, most studies (33, 35, 36) demonstrate AKT is sufficient for 4E-BP1 phosphorylation. Our results are similar to these previous studies, in that the dominant active E40K AKT we used was ineffective in activating p70 kinase activity or inducing p70 Thr\textsuperscript{389} phosphorylation but was capable of inducing 4E-BP1 phosphorylation. However, our dominant-interfering AKT mutant prevented both p70 activation as well as 4E-BP1 phosphorylation. Differences in the dominant negative AKT mutants used, vectors employed and cytokine stimulation (almost all prior studies analyze insulin stimulation) may account for the differences in our results when compared with previous studies (33, 37). In this regard, it is certainly possible that the inhibitory effect of our PH dominant negative AKT on p70 activation is the result of inhibition of a parallel pathway, possibly mediated by PDK1. If the PH construct inhibited PDK1 function, this would explain the prevention of p70 kinase activation as well as 4E-BP1 phosphorylation at Thr\textsuperscript{226} would be abrogated. In addition, recent evidence supports the ability of PDK1 to also phosphorylate p70 on Thr\textsuperscript{389} (38), which could also explain the ability of PH to inhibit p70 Thr\textsuperscript{389} phosphorylation.

Rapamycin was an effective inhibitor of p70 kinase activation, p70 phosphorylation, and 4E-BP1 phosphorylation, indicating the ability of mTOR to regulate all these events. mTOR can phosphorylate p70 at Thr\textsuperscript{389} in vitro (39, 40) but more recent studies (41–44) indicate that mTOR-mediated Thr\textsuperscript{389} phosphorylation and p70 activation occurs via an inhibition of protein phosphatase 2A-mediated dephosphorylation. Thus, rapamycin may inhibit Thr\textsuperscript{389} phosphorylation by activation of protein phosphatase 2A. The ability of rapamycin to also inhibit IL-6-dependent phosphorylation at Ser\textsuperscript{411} and Thr\textsuperscript{421}/Ser\textsuperscript{424} may also be caused by enhanced activity of this phosphatase because these residues in the auto-inhibitory domain are not substrates for mTOR in vitro (39). The rapamycin-induced inhibition of 4E-BP1 phosphorylation may also be the result of activation of a phosphatase or direct inhibition of mTOR kinase activity as mTOR can directly phosphorylate 4E-BP1 under some conditions (41). The inhibition of p70 activation and 4E-BP1 phosphorylation could explain the resulting cell cycle block induced by rapamycin. In addition, more recent work indicates rapamycin can induce cellular apoptosis, possibly because of inhibition of p70 phosphorylation of BAD (45). However, some cell types are resistant to the cytoreductive effects of rapamycin (29) for unclear reasons. To test the cellular effects of inhibiting mTOR function, we used rapamycin and its newly developed analogue, CCI-779. Although these drugs had no effect on the growth of AF-10 MM cells cultured without IL-6, they abrogated the IL-6 proliferative response in a dose-dependent fashion. Their lack of effect on unstimulated cells rules out nonspecific toxicity. Furthermore, the concentration of both drugs that curtailed IL-6-dependent cell growth effectively abrogated IL-6-dependent p70 and 4E-BP1 phosphorylation but had no effect on IL-6-dependent AKT activation (data not shown). As IL-6 is an important growth factor for tumor cells \textit{in vivo} in myeloma patients, targeting IL-6-mediated activation of p70 and phosphorylation of 4E-BP1 is a promising therapeutic modality.

REFERENCES

1. Anderson, K., Jones, R., Morimoto, C., Leavitt, P., and Barut, B. (1989) Blood \textbf{73}, 1915–1922
2. Lichtenstein, A., Tu, Y., Faddy, C., Vescio, R., and Berenson, J. (1995) \textit{Cell. Immunol.} \textbf{162}, 248–255
3. Bataille, R., Jeourdan, M., Zhang, X-G., and, Klein, B. (1989) \textit{J. Clin. Invest.} \textbf{84}, 2068–2071
4. Bataille, R., Barlogie, B., Lu, Z-Y., Rossi, J-P., Lavabre-Bertrand, T., Beck, T., Wijdenes, J., Brocherie, J., and Klein, B. (1995) \textit{Blood} \textbf{86}, 685–691
5. Hilbert, D., Kopf, M., Mock, B., Kohler, G., and Rudikoff, S. (1995) \textit{J. Exp. Med.} \textbf{182}, 243–244
6. Muraguchi, A., Hirano, T., Tang, B., Matsuda, T., HORI, Y., Nakajima, K., and Kishimoto, T. (1988) \textit{J. Exp. Med.} \textbf{167}, 332–341
7. Kishimoto, T., Akira, S., Naraasaki, M., and, Taga, T. (1995) \textit{Blood} \textbf{86}, 1243–1250
8. Ogata, A., Chauhan, D., Urashima, M., Teoh, G., Hatzizann, M., Vidiriales, V. M. B., Schlossman, R., and Anderson, K. (1997) \textit{J. Immunol.} \textbf{159}, 2212–2220
9. Daeipour, M., Kumar, G., Amaral, M. C., and Nel, A. (1993) \textit{J. Immunol.} \textbf{150}, 4743–4750
10. Cattell-Falcone, R., Landowski, T., Oshiro, M. M, Turkson, J., Levitzki, A.,
Signal Pathways Involved in Activation of p70S6K and Phosphorylation of 4E-BP1 following Exposure of Multiple Myeloma Tumor Cells to Interleukin-6
Yijing Shi, Jung-hsin Hsu, Liping Hu, Joseph Gera and Alan Lichtenstein

J. Biol. Chem. 2002, 277:15712-15720.
doi: 10.1074/jbc.M200043200 originally published online February 28, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M200043200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 44 references, 36 of which can be accessed free at http://www.jbc.org/content/277/18/15712.full.html#ref-list-1