Activation of the p70 S6 Kinase and Phosphorylation of the 4E-BP1 Repressor of mRNA Translation by Type I Interferons*

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The Type I IFN receptor-generated signals required for initiation of mRNA translation and, ultimately, induction of protein products that mediate IFN responses, remain uncertain. We have previously shown that IFNα and IFNβ induce phosphorylation of insulin receptor substrate proteins and downstream engagement of the phosphatidylinositol (PI) 3′-kinase pathway. In the present study we provide evidence for the existence of a Type I IFN-dependent signaling cascade activated downstream of PI 3′-kinase, involving p70 S6 kinase. Our data demonstrate that p70 S6K is rapidly phosphorylated on threonine 421 and serine 424 and is activated during treatment of cells with IFNα or IFNβ. Such activation of p70 S6K is blocked by pharmacological inhibitors of the PI 3′-kinase or the FKBP 12-rapamycin-associated protein mTOR. Consistent with this, the Type I IFN-dependent phosphorylation/activation of p70 S6K is defective in embryonic fibroblasts from mice with targeted disruption of the p85α and p85β subunits of the PI 3′-kinase (p85α−/−β−/−). Treatment of sensitive cell lines with IFNα or IFNβ also results in phosphorylation/inactivation of the 4E-BP-1 repressor of mRNA translation. Such 4E-BP1 phosphorylation is also PI3′-kinase-dependent and rapamycin-sensitive, indicating that the Type I IFN-inducible activation of PI3′-kinase and FRAP/mTOR results in dissociation of 4E-BP1 from the eukaryotic initiation factor-4E (eIF4E) complex. Altogether, our data establish that the Type I IFN receptor-activated PI3′-kinase pathway mediates activation of the p70 S6 kinase and inactivation of 4E-BP1, to regulate mRNA translation and induction of Type I IFN responses.

Type I interferons (IFNs) are pleiotropic cytokines that exhibit multiple biological effects on cells and tissues, including inhibition of cell proliferation of normal and malignant cells, induction of antiviral responses, as well as immunomodulatory activities (1–7). Several signaling pathways are activated following binding of Type I IFNs to the multichain Type I interferon receptor complex, whose IFNaR1 and IFNaR2 subunits are constitutively associated with protein members of the Jak family of kinases (reviewed in Refs. 1–7). A major Type I IFN-activated cellular pathway is the Jak-STAT signaling cascade (1–7). Engagement of the Type I IFN receptor results in activation of the Tyk-2 and Jak-1 kinases, which in turn regulate downstream phosphorylation/activation of the STAT1 and STAT2 transcriptional activators. The phosphorylated forms of STAT1 and STAT2 associate with IRF-9 (p48) to form the mature ISGF3 DNA-binding complex that translocates to the nucleus and regulates gene transcription via binding to ISRE elements in the promoters of IFN-stimulated genes (ISGs) (1–5). Several other STAT complexes are also induced during engagement of the Type I interferon receptor. STAT 1:1 homodimers, STAT 3:3 homodimers, STAT 1:3 heterodimers, STAT 5:5 homodimers, and CrkL:STAT5 heterodimers are formed in a Type I IFN-dependent manner and translocate to the nucleus where they bind to GAS regulatory elements in the promoters of IFN-activated genes to regulate gene transcription (2–5, 8, 9). In addition to tyrosine phosphorylation of STAT proteins by interferon-activated Jak kinases, phosphorylation on serine residues is required for their full transcriptional activation (10–14). It appears that, at least in the case of STAT1, phosphorylation on serine 727 is regulated by a member of the protein kinase C family of proteins, protein kinase C δ (15). There is also accumulating evidence that the p38 MAPK pathway is activated in a Type I IFN-dependent manner (16, 17) and that its function is essential for gene transcription via ISRE (16, 17) or GAS elements (18). Such regulatory effects of IFNs are pleiotropic.
this pathway play critical roles in IFN signaling, because p85 activation is essential for generation of Type I IFN-dependent antiproliferative responses (19–21).

The p70 S6 kinase was originally identified as a kinase that regulates serine phosphorylation of the 40 S ribosomal S6 protein (22–29). This kinase plays important roles in the regulation of cell-cycle progression, cell survival, as well as regulation of mRNA translation via phosphorylation of the 40 S ribosomal S6 protein (22–35). Previous studies have established that the activation of this kinase is regulated by the FKBP 12-rapamycin-associated protein (FRAP/mTOR), whose activation is in turn regulated by the upstream activation of the phosphatidylinositol 3’-kinase pathway (36–41).

The signals generated by the Type I interferon receptor to ultimately regulate mRNA translation are not known. We have previously demonstrated that Type I IFNs activate the insulin receptor substrate (IRS)-PI 3’-kinase pathway in human and mouse cells (42–45) and that both the lipid (42) and serine (45) kinase activities of the p110 catalytic subunit of the PI 3’-kinase are activated during engagement of the Type I interferon receptor. In the present study we sought to determine whether the p70 S6 kinase is activated downstream of the PI 3’-kinase to mediate induction of Type I IFN responses. Our data demonstrate that the p70 S6 kinase is rapidly phosphorylated and activated during treatment of sensitive cell lines with IFNα or IFNβ. They also show that the IFNα-dependent phosphorylation/activation of the p70 S6 kinase is defective in mouse embryonic fibroblasts (MEFs) from p85 knockout mice and that both the lipid (42) and serine (45) kinase activities of the p110 catalytic subunit of the PI 3’-kinase are activated during engagement of the Type I interferon receptor. In the present study we sought to determine whether the p70 S6 kinase is activated downstream of the PI 3’-kinase to mediate induction of Type I IFN responses. Our data demonstrate that the p70 S6 kinase is rapidly phosphorylated and activated during treatment of sensitive cell lines with IFNα or IFNβ. They also show that the IFNα-dependent phosphorylation/activation of the p70 S6 kinase is defective in mouse embryonic fibroblasts (MEFs) from p85 knockout mice and that both the lipid (42) and serine (45) kinase activities of the p110 catalytic subunit of the PI 3’-kinase are activated during engagement of the Type I interferon receptor.

MATERIALS AND METHODS

Cells Lines and Reagents—Human recombinant IFNα2 was provided by Hoffmann-La Roche. Human recombinant consensus IFNα was provided by Amgen Inc. Human recombinant IFNβ was provided by Biogen Inc. Antibodies against the phosphorylated forms of p70 S6 kinase, mTOR, and 4E-BP1 were obtained from Cell Signaling Technology Inc. An antibody against 4E-BP1 has been previously described (46). The FRAP/mTOR inhibitor, rapamycin, and the 3’-kinase inhibitors LY294002 and wortmannin were obtained from Calbiochem Inc. (La Jolla, CA). U266 cells were grown in RPMI 1640 supplemented with fetal bovine serum and antibiotics. U2OS and T98G cells were grown in McCoy’s and Dulbecco’s modified Eagle’s media, respectively, supplemented with fetal bovine serum and antibiotics. The generation of pS54a−/−β−/− mice, by crossing pS54a+/− mice with pS54a−/− mice (47) will be described elsewhere.2 The pS54a−/− mice embryonic fibroblasts were obtained from pS54a−/−β−/− double knockout mice. Briefly, mouse embryos were harvested at day 14; the limbs, head, and liver removed, and the resultant torso was finely minced. Following trypsinization, the single cell suspension was transferred onto gelatinized tissue culture dishes, and the mouse embryonic fibroblasts were immortalized after a few passages using SV40 large T antigen, expressed by a retrovector virus. The genotypes of the cells were determined by polymerase chain reaction. All transfections were performed using FuGENE 6, according to the manufacturer’s instructions (Roche Applied Science).

Cell Lysis and Immunoblotting—Cells were stimulated with 1 × 104 units/ml of the indicated IFNs for the indicated times, then lysed in phosphorylation lysis buffer as previously described (49). Immunoprecipitations and immunoblotting, using an enhanced chemiluminescence (ECL) method, were performed as previously described (49). In the experiments in which pharmacological inhibitors of FRAP/mTOR or the PI 3’-kinase were used, the cells were pretreated for 60 min with the indicated concentrations of the inhibitors and subsequently treated for the indicated times with IFNs, prior to lysis in phosphorylation lysis buffer. In some of the experiments to determine the phosphorylation of 4E-BP1, cell extracts were obtained by three freeze-thaw cycles, as previously described (46).

Isolation of Normal Peripheral Blood Granulocytes—Informed consent was obtained from healthy volunteers, according to the guidelines established by the Institutional Review Board of Northwestern University. Polymorphonuclear leukocytes were separated from peripheral venous blood using the Mono-Poly resolution medium (3M-PRM, ICN Biomedicals, Aurora, OH), as previously described (20). Briefly, after centrifugation at 300 g for 30 min, the plasma and the mononuclear leukocyte band were discarded, and the polymorphonuclear band was transferred into an individual tube. Cells were washed with culture medium and were subsequently resuspended in culture medium, prior to interferon treatment.

Luciferase Reporter Assays—Cells were transfected with a β-galactosidase expression vector and either an ISRE luciferase construct or a luciferase reporter gene containing eight GAS elements linked to a minimal prolactin promoter (8X-GAS), using the SuperFect transfection reagent as per the manufacturer’s recommended procedure (Qiagen). The ISRE-luciferase construct (16) included an ISG15 ISRE (TTTTGGATTTTGATCTGC) representing a cis-inducing element (SIE) of the c-fos promoter, was synthesized and used in the gel shift assays. A double-stranded oligodeoxynucleotide (CCTTGTGGTTTCTGGCCTCAGA), representing an ISRE element from the ISG-15 gene, was also synthesized and used to detect ISGF3 complexes.

RESULTS

The p70 S6 Kinase Is Activated by Type I Interferons Downstream of the Phosphatidylinositol 3’-Kinase—We initially sought to determine whether the p70 S6 kinase is phosphorylated/activated during treatment of Type I IFN-sensitive cell lines with IFNα or IFNβ. Molt-4 cells were incubated for 30 min in the presence or absence of IFNα. Cells were lysed in phosphorylation lysis buffer, and total cell lysates were analyzed by SDS-PAGE and immunoblotted with an antibody against the phosphorylated form of p70 S6 kinase on threonine 421 and serine 424. IFNα treatment resulted in strong phosphorylation of the p70 S6 kinase, whereas there was no change in the amounts of p70 S6 kinase protein detected after IFNα.
stimulation (Fig. 1, A and B). Similarly, phosphorylation of p70 S6K was detectable when another Type I IFN, IFNβ, was used (Fig. 1, C and D). In experiments, in which the phosphorylation of the p70 S6 kinase by Type I IFNs was determined, we found that both IFNα (Fig. 2, A and B), as well as IFNβ (Fig. 2, C and D) are capable of inducing phosphorylation of the protein when used at doses as low as 100 IU/ml, further emphasizing the specificity of the process. Furthermore, the Type I interferon-dependent phosphorylation of the p70 S6 kinase was inducible in various cell types, including U2OS osteosarcoma cells (Fig. 3, A and B) or U266 multiple myeloma cells (Fig. 2A, 2B, 3C, and 3D).

Phosphorylation of the S6 kinase was rapid, occurring within 15 min of IFN treatment and was still detectable after 120 min of incubation with IFN (Fig. 3, A and B). As expected, phosphorylation of p70 S6K was also detectable in lysates from cells treated with insulin, used as positive controls for these assays (Fig. 3, A–D). To examine whether the phosphorylation of p70 S6K also occurs in primary human cells, we determined whether IFNα is capable of inducing phosphorylation of the protein in human granulocytes isolated from the peripheral blood of healthy donors. Consistent with the data observed in cell lines, IFNα treatment induced p70 S6K phosphorylation in normal granulocytes (Fig. 3, E and F), suggesting that this kinase is also activated in primary human cells, and may participate in the induction of IFN responses under physiological conditions.

As our data established that p38 is phosphorylated during engagement of the Type I IFN receptor, we sought to identify the upstream signaling events that ultimately lead to p70 S6K activation. In previous studies we have shown that the serine and lipid kinase activities of the phosphatidylinositol 3-kinase are activated in a Type I IFN-dependent manner, during the interaction of the p85 regulatory subunit of this kinase with IRS proteins (IRS-1 and IRS-2) (42–45). Because the PI 3'-kinase pathway is a known regulator of activation of the p70 S6K, we examined whether its activation is required for engagement of the p70 S6 kinase in Type I interferon signaling.

Cells were pretreated with the PI 3'-kinase inhibitors LY294002 or wortmannin and were subsequently incubated in the presence or absence of IFNα. Treatment of cells with insulin was included as a positive control. As shown in Fig. 4, inhibition of PI 3'-kinase activity, by either LY294002 or wortmannin, abrogated the IFNα-dependent phosphorylation of p70 S6K (Fig. 4, A and B). Similarly, and as expected, the PI 3'-kinase inhibitors also blocked the insulin-dependent phosphorylation of the protein (Fig. 4, A and B).

To definitively establish the requirement of PI3'-kinase in the Type I IFN-dependent activation of p70 S6K, we undertook studies using embryonic fibroblasts from mice with targeted disruption of the genes for both the α and β isoforms of the p85 regulatory subunit of the PI 3'-kinase. p85α−/− p85β−/− or control p85α+/+ p85β+/+ MEFs were treated with mouse IFNα, and the phosphorylation of p70 S6K was examined by anti-phospho-p70 S6K immunoblotting. As shown in Fig. 5, IFNα treatment resulted in phosphorylation of p70 S6K on threonine 421 and serine 424 in p85α−/− p85β−/− cells but not in MEFs lacking expression of the p85 isoforms (p85α−/− p85β−/−) (Fig. 5, A and B). In a similar manner, when p85α−/− p85β−/− cells were compared with p85α+/− p85β−/− cells, phosphorylation of p70 S6K was detectable in the cells expressing p85α but not in the cells lacking both isoforms of the p85 regulatory subunit of the PI 3'-kinase (Fig. 5, C and D).

Thus, activation of the PI 3'-kinase pathway is essential for downstream engagement of the p70 S6K by Type I interferons, and the presence of either the p85α or p85β isoform of the PI 3'-kinase may be sufficient to mediate such a response.

**FRAP/mTOR Is Activated in a Type I IFN-dependent Manner to Regulate Downstream p70 S6 Kinase Activation**—The
IFNα Activates the p70 S6 Kinase

and, after cell lysis, total lysates were resolved by SDS-PAGE and immunoblotted with an anti-phospho-mTOR antibody. Some baseline phosphorylation of mTOR was detectable prior to IFN treatment (Fig. 6A). However, type I IFN treatment of the cells resulted in phosphorylation/activation of mTOR, demonstrating that this protein is indeed engaged in IFN-signaling (Fig. 6A).

The demonstration that the p70 S6 kinase is phosphorylated by type I IFNs strongly suggested that such phosphorylation activates the kinase domain of p70 S6K to regulate generation of IFN responses. We therefore sought to directly determine whether type I IFN-dependent treatment of cells results in induction of p70 S6 kinase activity. U-266 cells were incubated in the presence or absence of IFNα, and, after immunoprecipitation of cell lysates with an anti-p70 S6K antibody, immunoprecipitates were subjected to an in vitro kinase assay (52). As shown in Fig. 6B, type I IFN treatment resulted in an activation of the catalytic domain of the p70 S6 kinase (Fig. 6B). Such activation was blocked by pretreatment of cells with the PI 3'-kinase inhibitor LY294002, as well as by the FRAP/mTOR inhibitor rapamycin, demonstrating that the IFN-dependent induction of p70 S6 kinase activity is FRAP/mTOR-dependent (Fig. 6B). Consistent with this, the IFN-dependent phosphorylation of p70 S6 kinase was also blocked when cells were pre-treated with rapamycin prior to IFNα stimulation (Fig. 6C, D), confirming that p70 S6K is a downstream effector for mTOR. The effects of rapamycin (Fig. 6C) on p70 S6K phosphorylation were apparently due to suppression of the IFN-dependent phosphorylation and not the minimal baseline phosphorylation of the protein, because they were accompanied by suppression of the IFN-dependent kinase activity of the protein (Fig. 6B).

Activation of the p70 S6 Kinase Downstream of mTOR Does Not Regulate Type I IFN-dependent Gene Transcription—Our data demonstrating activation of p70 S6K downstream of FRAP/mTOR, indicated that this pathway plays a role in mRNA translation, because it is well established that the kinase activity of p70 S6K regulates phosphorylation of the 40S ribosomal S6 protein. We also examined whether activation of FRAP/mTOR downstream of the PI 3'-kinase plays any role in the type I IFN-inducible activation of the STAT-pathway, which regulates transcriptional regulation of IFN-sensitive genes. We initially performed gel mobility shift assays, using either ISRE or a GAS (SIE) recognition element, to evaluate whether FRAP/mTOR inhibition on the formation of STAT-binding complexes. Cells were incubated with IFNα in the presence or absence of rapamycin, and the formation of the active ISGF3 or SIF complexes in response to IFNα was determined. As expected, treatment of various cell lines with IFNα induced formation of ISGF3 complexes (STAT2:STAT1:IRF-9) (Fig. 7A) or SIF complexes (STAT3:3, STAT1:3, STAT1:1) (Fig. 7, B–D). Rapamycin did not block the induction of ISGF3 or SIF complexes (Fig. 7, A–D), indicating that the activation of the p70 S6 kinase does not exhibit regulatory effects upon STAT activation. In a similar manner, treatment of cells with rapamycin had no effects on the phosphorylation of STAT proteins or their DNA binding activities in vitro (16, 18). Our data demonstrated that the function of the p70 S6 kinase is not

FKB12-rapamycin associated protein (FRAP), also called mammalian target of rapamycin (mTOR), has previously been shown to regulate activation of the p70 S6 kinase, downstream of the PI 3'-kinase and the PDK-1 kinase (36–41, 55). It is also well established that activation of mTOR requires its phosphorylation on serine 2448. We investigated whether mTOR is engaged in type I IFN signaling, to regulate downstream activation of the p70 S6K. U-266 cells were treated with IFNβ,
required for STAT activation and their DNA-binding activities but did not exclude the possibility that this pathway may still be facilitating transcriptional regulation of IFN-sensitive genes via effects on auxiliary pathways, including the p38 pathway. To address this issue, experiments were performed in which the effects of rapamycin on gene transcription via ISRE or GAS elements were examined. Cells were transfected with ISRE or 8X-GAS luciferase constructs and treated with IFNα, in the presence or absence of the FRAP/mTOR inhibitor rapamycin. Luciferase activity was subsequently measured. As expected, IFNα induced strong luciferase activity via either ISRE (Fig. 8A) or GAS elements (Fig. 8B), but preincubation with rapamycin had no effect on the induction of such luciferase activities. Thus, based on these findings, it is unlikely that activation of p70 S6K downstream of PI 3'-kinase and that this phosphorylation inhibits its activity, we examined the effects of IFNα-dependent gene transcription, and its primary role in IFN signaling appears to be mediation of signals that regulate mRNA translation.

**Type I IFN-dependent Phosphorylation of the 4E-BP1 Repressor of mRNA Translation and Its Dissociation from the Eukaryotic Initiation Factor-4E (eIF4E) Complex**—It has previously been shown that, in response to insulin, the 4E-BP1 repressor of mRNA translation is phosphorylated downstream of the PI 3'-kinase pathway and that this phosphorylation inhibits its interaction with the initiation factor eIF4E (46). We sought to determine whether IFNα treatment also results in phosphorylation of 4E-BP1. U-266 cells were treated with IFNα, and after cell lysis, total lysates were analyzed by SDS-PAGE and immunoblotted with an antibody against the phosphorylated form of p70 S6 kinase. The blot shown in A was stripped and re-probed with an anti-p70 S6 kinase antibody, to control for protein loading. C, p85α−/−β−/− and p85α+/+β+/+ immortalized mouse embryonic fibroblasts (MEFs) were treated with mouse IFNα for the indicated times. Total cell lysates were analyzed by SDS-PAGE and immunoblotted with an antibody against the phosphorylated form of p70 S6 kinase. B, the blot shown in A was stripped and re-probed with an anti-p70 S6 kinase antibody, to control for protein loading.

As shown in Fig. 9B, treatment with LY294002 or rapamycin abrogated the IFNα-inducible hyperphosphorylated form of 4E-BP1 (Fig. 8B), indicating that this event occurs downstream of PI 3'-kinase/mTOR activation. On the other hand, the SB203580 inhibitor, which selectively blocks activation of the p38 MAPK (16–18), had no effects on 4E-BP1 phosphorylation (Fig. 9B). To further confirm the phosphorylation of 4E-BP1 in response to Type I IFNs, experiments were performed in which the phosphorylation of the 4E-BP1 protein was detected by using an anti-phospho 4E-BP1 antibody that recognizes the protein only when it is phosphorylated on threonine 70. Using this approach, strong IFNα-dependent phosphorylation of 4E-BP1 was detectable in U-266 cells (Fig. 10, A and B) as well as COS cells (Fig. 10, C and D), whereas this phosphorylation was inhibited by pretreatment of cells with rapamycin or LY294002 (Fig. 10, A and B).

Recent studies have shown that, in addition to phosphorylation on Thr-70, phosphorylation of additional sites, most likely the priming sites Thr-37 and Thr-46, is essential for the release of 4E-BP1 from eIF4E (60). We examined whether Type I IFN treatment induces phosphorylation of these sites, whose function is critical for optimal phosphorylation of 4E-BP1. Cells were incubated in the presence or absence of IFNβ, and total cell lysates were analyzed by SDS-PAGE and immunoblotted with an anti-4E-BP1 antibody (46, 54). IFNα treatment resulted in a mobility shift of the 4E-BP1 protein and detection of a slowly migrating form (Fig. 9A), which corresponds to the hyperphosphorylated form of the protein (46, 54). To determine whether such phosphorylation of 4E-BP1 requires upstream activation of the PI 3'-kinase and FRAP/mTOR activity, we examined the effects of LY294002 and rapamycin on such phosphorylation. Cells were preincubated with the different pharmacological inhibitors and subsequently treated with IFNα. The phosphorylation of 4E-BP1 was subsequently analyzed by determination of the mobility shift of the protein. As shown in Fig. 9B, treatment with LY294002 or rapamycin abrogated the IFNα-inducible hyperphosphorylated form of 4E-BP1 (Fig. 8B), indicating that this event occurs downstream of PI 3'-kinase/mTOR activation. On the other hand, the SB203580 inhibitor, which selectively blocks activation of the p38 MAPK (16–18), had no effects on 4E-BP1 phosphorylation (Fig. 9B). To further confirm the phosphorylation of 4E-BP1 in response to Type I IFNs, experiments were performed in which the phosphorylation of the 4E-BP1 protein was detected by using an anti-phospho 4E-BP1 antibody that recognizes the protein only when it is phosphorylated on threonine 70. Using this approach, strong IFNα-dependent phosphorylation of 4E-BP1 was detectable in U-266 cells (Fig. 10, A and B) as well as COS cells (Fig. 10, C and D), whereas this phosphorylation was inhibited by pretreatment of cells with rapamycin or LY294002 (Fig. 10, A and B).
addition to the activation of the p70 S6 kinase, Type I IFN-dependent activation of PI 3-kinase and FRAP/mTOR regulates phosphorylation of 4E-BP1 and its dissociation from eIF4E, providing an additional mechanism by which this pathway regulates mRNA translation in response to Type I IFNs.

**DISCUSSION**

Type I IFNs exhibit multiple biological activities, including antiproliferative, antiviral, and immunomodulatory effects *in vitro* and *in vivo* (1–7). The importance of the biological properties of Type I IFNs has led to extensive studies to understand the mechanisms by which these cytokines exert their biological effects on target cells. Over the last few years, dramatic advances have occurred in our understanding of how signals generated at the Type I IFN receptor level ultimately result in the induction of the effects of interferons. It is now well documented that the signals generated upon binding of IFNα or IFNβ to the Type I IFN receptor result in the production of proteins that exhibit antiviral and/or antiproliferative properties (1–7). The transcription of genes encoding for such proteins is regulated by activation of Type I IFN-dependent Jak-STAT pathways (1–4). Such transcription appears to be facilitated by STAT proteins that exhibit antiviral and/or antiproliferative properties (1–7).

In addition to the Jak-STAT and p38 MAPK signaling cascades, Type I interferons engage insulin receptor substrate (IRS) proteins, to regulate activation of the PI 3'-kinase (42–45, 58). This pathway is activated by all Type I IFNs (42) downstream of Jak kinases (43, 58), and there is evidence that it mediates classic IFN responses, including protection from...
viral infection (59). However, the precise mechanisms that ultimately lead to the generation of IFN biological effects through this signaling cascade are not known. It is well established that interferons regulate gene transcription and mRNA translation for target genes in a variety of malignant and non-malignant cells, resulting in the production of various protein products, among which proteins that suppress neoplastic cell proliferation, such as the pml gene product (60–63). On the other hand, it is well established that one mechanism by which interferons mediate antiviral responses is suppression of viral replication for different viruses, via regulation of viral RNA translation (64–69). In the present study we provide evidence that activation of the PI 3'-kinase by Type I IFNs ultimately leads to activation of the p70 S6 kinase, phosphorylation of the 4E-B1 repressor of mRNA translation, and its dissociation from the eIF4E. These data provide the first direct evidence linking an interferon-signaling pathway to events that positively regulate translation for target genes in a variety of malignant and non-malignant cells.

Several different cascades have been previously shown to be activated downstream of the phosphatidylinositol 3'-kinase in eukaryotic cells. It is now well established that signaling proteins with pleckstrin homology domains bind directly to phosphatidylinositol 3,4,5-trisphosphate, which results from conversion of the plasma membrane phosphatidylinositol 4,5-bisphosphate by the activated PI 3'-kinase (reviewed in Ref. 69).

These include the kinase PDK1 and the Akt kinase (69), which activate diverse downstream signaling cascades. PDK1 phosphorylates and activates the Akt kinase (69), which in turn activates several downstream effectors, including the mammalian target of rapamycin FRAP/mTOR (70, 76, 77), which subsequently mediates phosphorylation of p70 S6K...
that, in that study, addition of exogenous of IFNβ, in the presence of PI 3-kinase and mTOR inhibitors, did not restore NO production (78). Taken together with our data, these findings suggest that this pathway is part of an autocrine loop that regulates production of Type I IFNs and, most importantly, mediates Type I IFN signals. Such downstream signals appear to be activation of the p70 S6K and de-activation of 4E-BP1, which in the context of interferon-induced transcription, facilitate protein translation of mRNA for IFN-sensitive genes and, therefore, induction of IFN-dependent biological effects.

It remains to be determined whether other signaling elements and pathways, beyond the IRS-PI 3-kinase pathway, facilitate activation of p70 S6K and phosphorylation of 4E-BP1. It is well established that several sites of phosphorylation exist in 4E-BP1, and that the phosphorylation of the protein occurs via a two-step mechanism (79). Recently, it was shown that Erk kinases can phosphorylate 4E-BP1 in response to stress (80), whereas the downstream effector of the p38 MAPK, Ms1, phosphorylates 4E-BP1 in response to ultraviolet irradiation (81). Previous studies have also demonstrated that Type I IFNs activate Erk kinases (82) and that such activation is PI 3-kinase-dependent (45). Similarly, the p38 MAPK pathway is activated by Type I IFNs (16–18), and in recent studies we have observed that Ms1 is phosphorylated/activated in a Type I IFN-dependent manner.3 Most importantly, activation of the p38 MAPK pathway is essential for the generation of Type I IFN-dependent antiproliferative and/or antiviral responses in a variety of different cell types (19, 20). Thus, it is possible that these kinases are also required for phosphorylation/inactivation of 4E-BP1 and initiation of Type I IFN-dependent mRNA translation, but this remains to be examined in future studies.

Independently of the precise mechanisms involved, our data for the first time establish that the PI 3′-kinase pathway and cascades downstream of FRAP/mTOR participate in Type I IFN signaling, to generate IFN-dependent translational responses. These findings may have important clinical implications, because there are ongoing efforts toward the clinical development of rapamycin and the related analog CCI-779 for the treatment of certain malignancies (56, 57). Such efforts are conceptually based on the documented ability of these compounds to inhibit growth factor-dependent malignant cell growth. IFNα is an antitumor agent widely used in clinical oncology and clinical virology. Therefore, our data indicate that caution should be taken in designing clinical trials combining these agents with IFNs, because it is possible that they may ameliorate its antitumor properties in vivo.

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FIG. 11. Type I IFN-dependent phosphorylation of 4E-BP1 and its dissociation from eIF4E. U-266 cells were incubated in the presence or absence of IFNα the indicated. Total cell lysates were analyzed by SDS-PAGE and were immunoblotted with antibodies against 4E-BP1 or with a phospho-antibody that recognizes the phosphorylated form of 4E-BP1 on threonines 37 and 46. A, 4E-BP1 immunoblot is shown. B, anti-4E-BP1 immunoblots of the same blot shown in A is shown. C and D, U-266 cells were incubated in the presence or absence of IFNα, for 90 min, as indicated. Cells were lysed by freeze-thaw cycles, and total extracts were either analyzed directly by SDS-PAGE or incubated with m7GDP-agarose resin prior to SDS-PAGE analysis. C, equal amounts of total cell extracts were resolved by SDS-PAGE and immunoblotted with an anti-4E-BP1 antibody. D, proteins bound to the m7GDP-agarose resin were eluted, analyzed by SDS-PAGE, and immunoblotted with an anti-4E-BP1 antibody.

(36–41) and 4E-BP1 (71–74). Thus, FRAP/mTOR is required for the phosphorylation/activation of the p70 S6 kinase and phosphorylation/inactivation of 4E-BP1, events essential for the initiation of protein translation. Other pathways activated downstream of Akt include forkhead-related transcription factor 1, the inducer of apoptosis, and the glycogen synthase kinase 3 (70, 76, 77).

The PI 3′-kinase-dependent pathways are traditionally believed to be pathways that mediate events essential for cell growth and anti-apoptotic effects. In fact, most studies have focused on the role of these proteins in growth factor signaling or in the context of malignant transformation by oncoproteins. Our finding, that Type I IFNs also activate the S6 kinase and phosphorylate 4E-BP1, in a FRAP/mTOR-dependent manner, implicates these proteins in the generation of antiproliferative and/or antiviral responses. Thus, it is likely that, as in the case of other pathways (Jak-STAT and MAPK cascades), the PI 3′-kinase/FRAP/mTOR pathway is capable of mediating either cell-proliferative or growth inhibitory responses, depending on the stimulus and the cellular context. This is not surprising, because it is well established that Type IFNs regulate transcription of genes, whose protein products suppress cell growth and mediate antiviral responses. Interestingly, a previous study (78) had demonstrated that PI 3′-kinase and mTOR mediate lipopolysaccharide-stimulated nitric oxide (NO) production in macrophages via secretion of IFNβ, which functions as an autocrine cofactor for NO production (78). It is of interest...
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Activation of the p70 S6 Kinase and Phosphorylation of the 4E-BP1 Repressor of mRNA Translation by Type I Interferons

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