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Dual Regulatory Roles of Phosphatidylinositol 3-Kinase in IFN Signaling

Surinder Kaur,* Antonella Sassano,* Ajith M. Joseph,* Beata Majchrzak-Kita,† Elizabeth A. Eklund,* Amit Verma,‡ Saskia M. Brachmann,§ Eleanor N. Fish,† and Leonidas C. Platanias‡*

PI3K is activated by the type I and II IFN receptors, but its precise role in the generation of IFN responses is not well understood. In the present study we used embryonic fibroblasts from mice with targeted disruption of the genes encoding for both the p85α and p85β regulatory subunits of PI3′-kinase (p85α−/−β−/−) to precisely define the role of PI3K in the control of IFN-induced biological responses. Our data demonstrate that PI3K plays dual regulatory roles in the induction of IFN responses by controlling both IFN-α- and IFN-γ-dependent transcriptional regulation of IFN-sensitive genes and simultaneously regulating the subsequent initiation of mRNA translation for such genes. These processes include the Isg15, Cxcl10, and/or Ifi27 genes, whose functions are important in the generation of the biological effects of IFNs. Consistent with this, the induction of IFN antiviral responses is defective in double p85α/p85β knockout cells. Thus, integration of signals via PI3K is a critical event during engagement of the IFN receptors that complements both the transcriptional activity of Jak-STAT pathways and controls initiation of mRNA translation. The Journal of Immunology, 2008, 181: 7316–7323.

FNs are cytokines that play critical roles in the host defense against viral and parasitic infections, as well as in immune-surveillance against malignant cells (1–6). Because of these properties, IFNs have been used in the treatment of viral infections as well as various malignancies and neurological disorders (7–10). Over time, there has been an increasing interest in understanding the signaling mechanisms downstream of the IFN receptors, since insights into the cellular events that mediate the antiviral and antitumor effects of IFNs may lead to the design of novel drugs that target specific signaling effectors. Among the signaling cascades and elements that transmit type I and II IFN signals, the Jak-STAT pathway has been the most extensively studied. Different combinations of Jak kinases are associated with the different IFN receptors, and binding to these cell surface receptors results in activation of Jak and downstream phosphorylation/activation of the STATs. The activated STATs then translocate to the nucleus and trigger transcription of IFN-stimulated genes (ISGs)4 via binding to specific elements in the promoters of the ISGs (1–6).

Distinct from the classic Jak-STAT pathways, multiple other signaling cascades are activated by IFNs, underscoring the complexity of the signaling mechanisms invoked by IFNs (11–19). For example, MAPK pathways and, in particular, the p38 MAPK signaling pathway are activated by type I IFNs and are required for full transcriptional activation of ISGs and IFN-generated biological responses, separate from STAT activation (11–15). Another major signaling pathway activated by IFNs is the PI3K pathway. In mammals, IFNs and other cytokines engage class I PI3Ks, which exist as inactive heterodimers of the regulatory (p85) and the catalytic (p110) subunits in the cytoplasm of cells (20). The p85 regulatory subunit is recruited to the cell membrane by activated receptor tyrosine kinases, and this induces a conformational change in the complex, resulting in reversal of the inhibitory effects on the p110 catalytic subunit and activation of its kinase domain (20). Adapters such as the insulin receptor substrate (IRS) proteins serve as docking proteins to regulate downstream engagement of PI3K in response to IFNs (21–23).

In previous work, we demonstrated that activation of PI3K by type I and II IFNs mediates activation of p70S6K and phosphorylation/deactivation of the translational repressor eukaryotic initiation factor 4E-binding protein (4E-BP)1 (24–26). These studies have raised the possibility that PI3K participates in the regulation of mRNA translation in response to IFNs, but its precise role in mRNA translation for ISGs has not been directly established. In the present study we used mouse embryonic fibroblasts (MEFs) with targeted disruption of the genes for both the p85α and p85β regulatory subunits of PI3K to study its precise role in the generation of IFN responses. Collectively, our data establish that PI3K...

4 Abbreviations used in this paper: ISG, IFN-stimulated gene; ECMV, encephalomyocarditis virus; 4E-BP, eukaryotic initiation factor 4E-binding protein; GAS, IFN-γ activated site; IRS, insulin receptor substrate; ISRE, IFN stimulated response element; MEF, mouse embryonic fibroblast; mTOR, mammalian target of rapamycin.
exhibits dual regulatory functions in IFN signaling, controlling both transcription and mRNA translation of IFN-inducible genes, and plays a critical role in the generation of the antiviral effects of IFNs.

Materials and Methods

Cell lines and reagents

Recombinant mouse IFN-α4 was provided by Wellcome Research Laboratories. Recombinant mouse IFN-β was provided by Dr. Darren Baker (Biogen Idec). Recombinant mouse IFN-γ was from PBL Biomedical Laboratories. Immortalized MEFs from p85 knockout mice and the retroviral add-back MEFs have been described previously (24, 25, 27). The MEFs were cultured in DMEM containing 10% FCS and antibiotics. A rabbit polyclonal Ab against mouse ISG15 was kindly provided by Dr. Dong-Er Zhang (Scripps Research Institute, La Jolla, CA). Another polyclonal Ab against ISG15 has been previously described (28). Abs against tubulin and IP10 were from Abcam. Abs against of Akt, 4E-BP1, and their phosphorylated forms were from Cell Signaling Technologies.

Cell lysis and immunoblotting

For short-time treatments (up to 60 min), cells were starved overnight in serum-free medium. Cells were treated with the indicated IFNs for the indicated times and lysed in hypotonic lysis buffer supplemented with PMSF, aprotinin, and orthovanadate, as previously described (25, 26, 28). Equal protein aliquots were resolved by SDS-PAGE, and immunoblotting using an ECL method was performed as in our previous studies (24–26, 28).

Luciferase reporter assays

Luciferase reporter assays were performed as previously described (12, 26, 28). The ISRE (IFN stimulated response element) luciferase construct (29) was provided by Dr. Richard Pine (Public Health Research Institute, New York, NY), while the 8× GAS (IFN-γ activated site) construct (30) was provided by Dr. Christofer Glass (University of California, San Diego, CA). Cells were transfected with a β-galactosidase construct and either ISRE or 8× GAS luciferase constructs using SuperFect (Qiagen). Forty-eight hour post-transfection cells were either left untreated or treated with IFN for 6 h and luciferase activity was measured. β-galactosidase activity was used for normalization (12, 26, 28).

Quantitative RT-PCR (TagMan)

Cells were treated with 5000 IU/ml of IFN-α or 2500 IU/ml of IFN-γ for 6 h and RNA was isolated using RNeasy kit (Qiagen). RNA (1 μg) was reverse-transcribed using Omniscript reverse transcriptase and oligo(dT) primer (Qiagen). Real-time RT-PCR for the Isg15 and Ipl10 genes was conducted on ABI7900 sequence detection system (Applied Biosystems) using commercially available FAM-labeled probes and primers (Applied Biosystems). GAPDH was used for normalization. ΔCt values (target gene Ct − GAPDH Ct) for each triplicate sample were averaged, and ΔΔCt was calculated. mRNA amplification was determined by formula 2−ΔΔCt, as previously described (28). Relative quantitation of mRNA levels was plotted as fold increase over untreated samples.

Isolation of polysomal RNA and quantitative RT-PCR

p85α−/−β−/− and p85α−/−β−/− MEFs were treated with mIFN-α or mIFN-γ for 40 h, and isolation of polysomal RNA and quantitative RT-PCR on the polysomal fractions were performed as previously described (28). Polysomes were lysed in hypotonic lysis buffer supplemented with protease inhibitors (Calbiochem), RNase inhibitor (Ambion), 1 mM DTT, 100 g/ml cycloheximide. Triton X-100 and sodium deoxycholate were added to the lysates to a final concentration of 0.5% each. The lysates were clarified by centrifugation and supernatants were layered over 10–50% continuous sucrose gradient. After ultracentrifugation, fractions were collected monitoring the absorbance at 254 nm as a function of gradient depth. The polysomal fractions were pooled and total RNA from pooled fractions was isolated using RNAqueous-Micro kit from Ambion. Total polysomal RNA for each experimental condition was quantitated and equal amounts of RNA were reverse-transcribed into cDNA using the Omniscript RT kit and Oligo(dT) primers (Qiagen). Real-time PCR for the Isg15, Ipl7, or Ccc1l0 gene was conducted using commercially available FAM-labeled probes and primers (Applied Biosystems), and GAPDH was used for normalization. To further confirm the results, real-time PCR for the Isg15 and Ccc1l0 genes was also repeated using tubulin for normalization. mRNA amplification was determined by the formula 2−ΔΔCt as described above, and relative quantitation of mRNA levels was plotted as fold increase as compared with untreated samples (28).

Antiviral assays

The antiviral effects of mouse IFN-α were determined in assays using encephalomyocarditis virus (EMCV) as the challenge virus, as in our previous studies (12, 26, 28).

Results

The PI3K pathway is required for activation of Akt by IFNs

In initial studies, we sought to determine whether engagement of PI3K is required for downstream phosphorylation of Akt, whose function is required for activation of pathways that regulate initiation of mRNA translation by IFNs (28). For this purpose, we used MEFs derived from mice with targeted disruption of both the p85α and p85β subunits of the PI3K (p85α−/−β−−), p85α−/−β−/− and p85α−/−β−/− MEFs were treated with either mouse IFN-α (Fig. 1A) or mouse IFN-γ (Fig. 1B), and cell lysates were processed for immunoblotting with an Ab that recognizes the phosphorylated

![FIGURE 1. IFN-dependent phosphorylation/activation of Akt is PI3K dependent. A, p85α−/−β−/− and p85α−/−β−/− MEFs were treated with mouse IFN-α for the indicated times. The cells were lysed and equal amounts of protein were resolved by SDS-PAGE and immunoblotted with an Ab against phosphorylated form of Akt on Ser473 (top panel). The same blot was stripped and reprobed with an anti-Akt Ab (lower panel). B, p85α−/−β−/− and p85α−/−β−/− MEFs were treated with mouse IFN-γ for the indicated times. Equal amounts of protein were resolved by SDS-PAGE and immunoblotted with an Ab against phosphorylated form of Akt on Ser473 (top panel). The same blot was stripped and reprobed with an anti-Akt Ab (lower panel). C, p85α−/−β−/− or p85α−/−β−/− MEFs were treated with mouse IFN-γ for the indicated times. Equal amounts of protein were resolved by SDS-PAGE and immunoblotted with an Ab against the phosphorylated form of Akt on Thr308 (top panel). The same blot was stripped and reprobed with an anti-Akt Ab (lower panel).](http://www.jimmunol.org/DownloadedFrom/7317)
form of Akt on Ser473. Treatment of the p85α+/+ β+/+ and p85α−/− β−/− MEFs with either IFN-α or IFN-γ resulted in strong phosphorylation of Akt on Ser473, but such phosphorylation was defective in p85α−/− β−/− MEFs (Fig. 1, A and B). Similar results were also seen when the IFN-α-inducible phosphorylation of Akt on Thr308, the PDK1 phosphorylation site (31), was compared in p85α−/− β−/− and p85α−/− β−/− MEFs treated with mouse IFN-γ for the indicated times. Equal protein aliquots were resolved by SDS-PAGE and immunoblotted with Abs against the phosphorylated form of 4E-BP1 on Thr70 (top panel). The same blots were subsequently stripped and reprobed with an anti-4E-BP1 Ab (middle panel). The signals for phospho-4E-BP1 and 4E-BP1 or GAPDH controls from three independent experiments, including the experiment shown above, were quantitated by densitometry, and the intensity of phospho-4E-BP1 relative to 4E-BP1 or GAPDH controls was calculated. Data are expressed as the means of ratios of phospho-4E-BP1 to 4E-BP1 or GAPDH levels ± SE for each experimental condition (bottom panel).

In previous work, we had demonstrated that engagement of PI3K is required for type I and II IFN-induced phosphorylation of p70 S6 kinase (24, 25). Also, studies with the pharmacological inhibitors had suggested that phosphorylation and deactivation of translation repressor 4E-BP1 may occur downstream of PI3K and mammalian target of rapamycin (mTOR) (24–26). To definitively address this issue, we sought to determine whether IFN-induced phosphorylation of 4E-BP1 is defective in p85α−/− β−/− MEFs. As shown in Fig. 2, A and B, both type I and II IFN treatment
induced strong phosphorylation of 4E-BP1 in p85α+/β+/- MEFs on Thr70 (Fig. 2, A and B) or Thr37/46 (Fig. 2, C and D), but such phosphorylation was reduced but not entirely eliminated in p85α-/- β-/- MEFs (Fig. 2).

Regulation of IFN-inducible expression of IFN-stimulated gene products by PI3K

We next sought to determine the role PI3K in expression of protein products that are important for generation of the biological effects of IFNs. We examined the expression of ISG15, a type I IFN inducible protein that plays an important role in ISGylation of proteins and generation of antiviral responses (32, 33). Additionally, we studied the role of p85 in expression of CXCL10, a type II IFN-induced protein that plays a role in IFN-γ-induced apoptosis and inhibition of viral replication (34). p85α-/β+/- and p85α-/- β-/- MEFs were treated with mouse IFN-α or mouse IFN-β, and cell lysates were resolved by SDS-PAGE and immunoblotted with an anti-ISG15 Ab. There was strong induction of ISG15 in p85α+/β+/- MEFs, but such induction was defective in p85α-/- β-/- MEFs (Fig. 3, A and B), establishing a requirement for PI3K in the expression of ISG15 protein. Similarly, the IFN-γ-inducible expression of CXCL10 was defective in p85α-/- β-/- cells (Fig. 3C). To definitively establish the requirement of PI3K in IFN-inducible expression of ISG15, experiments were performed in which ISG15 induction was compared in p85α-/β-/- MEFs and p85α-/- β-/- MEFs, in which p85α or p85β was ectopically reexpressed. As shown in Fig. 4A, ectopic expression of p85α or p85β in p85α-/- β-/- cells partially restored the ability of IFN-α to induce ISG15 expression (Fig. 4A). Similarly, the ability of mouse IFN-β to induce ISG15 was also partially restored in cells complemented with p85α or p85β (Fig. 4B).

Collectively, our data established that PI3K activity is essential for induction of type I and II IFN-regulated gene products that play key roles in the generation of the biological effects of IFNs. To better understand the mechanisms by which such effects occur, we proceeded to determine whether such regulation occurs at the transcriptional or posttranscriptional levels or both. We first examined whether there are differences between wild-type MEFs and double p85α/p85β knockouts, in terms of IFN-inducible gene transcription via ISRE or GAS elements, in luciferase reporter assays. p85α+/β+/- or p85α-/- β-/- MEFs were transfected with an ISRE or 8x GAS luciferase construct, and after treatment with IFN-α or IFN-γ, luciferase activity was measured. As shown in Fig. 5, there was induction of luciferase activity in p85α+/β+/- MEFs in response to IFN-α and IFN-γ, but very little activity was seen in p85α-/- β-/- MEFs (Fig. 5, A and B), suggesting that defective gene transcription may account, in part, for the decreased expression of the ISG15 or CXCL10 proteins. Consistent with this, when gene transcription of the Isg15 and/or Cxcl10 genes was directly

FIGURE 3. Type I or II IFN-induced expression of ISG15 and CXCL10 is defective in the absence of the p85α and p85β subunits of PI3K. A, p85α+/β+/- or p85α-/- β-/- MEFs were treated with mouse IFN-α for 48 h and, after cell lysis, equal protein aliquots were resolved by SDS-PAGE and immunoblotted with an anti-ISG15 Ab (top panel). The same blot was reprobed with anti-tubulin Ab as a control for loading (lower panel). B, p85α+/β+/- or p85α-/- β-/- MEFs were treated with mouse IFN-β for 24 h and, after cell lysis, equal protein aliquots were resolved by SDS-PAGE and immunoblotted with an anti-ISG15 Ab (top panel). The same blot was reprobed with an anti-GAPDH Ab as a control for loading (lower panel). C, p85α+/β+/- or p85α-/- β-/- MEFs were treated with mouse IFN-γ for 48 h and, after cell lysis, equal protein aliquots were resolved by SDS-PAGE and immunoblotted with an anti-CXCL10 (IP10) Ab (top panel). The same blot was reprobed with anti-tubulin Ab as a control for loading (lower panel).

FIGURE 4. Restoration of ISG15 expression in p85α/p85β double-knockout MEFs by ectopic reexpression of the p85α or p85β. A, p85α+/β+/- or p85α-/- β-/- MEFs transfected with retroviral constructs encoding either p85α isoform (pMIG-p85α) or p85β isoform (pMIG-p85β) or with control vector alone (pMIG) were treated with mouse IFN-α as indicated. Equal protein aliquots were resolved by SDS-PAGE, and immunoblotting was done with an anti-ISG15 Ab (top panel). The blot was reprobed with anti-tubulin Ab to control for protein loading (lower panel). B, p85α+/β+/- or p85α-/- β-/- MEFs transfected with retroviral constructs encoding either the p85α isoform (pMIG-p85α) or p85β isoform (pMIG-p85β) or control vector alone (pMIG) were treated with mouse IFN-β as indicated. Equal protein aliquots were resolved by SDS-PAGE, and immunoblotting was done with an anti-ISG15 Ab (top panel). The same blot was reprobed with anti-GAPDH Ab as a control for protein loading (lower panel).
Polysomes isolated from IFN-α-treated p85α+/+β+/+ cells, but not p85α−/−β−/− cells (Fig. 6, B and C), establishing that mRNA translation for the Isg15 gene is defective in the absence of PI3K activity. Similarly, the polysomal distribution of Irf7 mRNA in p85α+/+β+/+ vs p85α−/−β−/− MEF cells was also examined. This assessment was particularly relevant, as Irf7 plays an important role in antiviral and immune responses and is a regulator of IFN production in innate immune responses (35–37), while recent studies have shown that the 5′ untranslated region of Irf7 is highly structured and its translation is de-repressed in 4E-BP1 and 4E-BP2 knockout mice (37). There was strong induction of Irf7 mRNA in polysomes isolated from IFN-α-treated p85α+/+β+/+ cells, but such an increase was not seen in p85α−/−β−/− cells (Fig. 6D). Similar results were also seen when p85α+/+β+/+ and p85α−/−β−/− MEFs were treated with mIFN-γ and the abundance of Cxcl10 mRNA in polysomal fractions was evaluated. There was increased expression of Cxcl10 polysomal mRNA in p85α+/+β+/+ MEFs, but such an increase was not seen in p85α−/−β−/− MEFs (Fig. 7). Taken together, these data established that mRNA translation of IFN-responsive genes is defective in cells with targeted disruption of both the p85α and p85β subunits of PI3K, underscoring the relevance of the PI3K pathway in the process.

To directly determine whether such defective gene transcription and mRNA translation seen in p85α−/−β−/− MEFs have consequences in the generation of the antiviral effects of IFN-α, the ability of IFN-α to inhibit replication of EMCV was assessed. As shown in Fig. 8, p85α+/+β+/+ MEFs were much more sensitive to the antiviral effects of IFNs as compared with p85α−/−β−/− MEFs, establishing an important regulatory role for PI3K in control of IFN-generated antiviral responses.

**Discussion**

Beyond the classical Jak-STAT pathways, IFNs activate several other signaling cascades in normal and malignant cells. Such pathways either complement the function of Jak-STAT pathways or mediate IFN-dependent biological responses independently of them (4, 7, 38, 39). Among the alternate pathways activated by IFNs, the PI3K pathway appears to play a prominent role in the generation of biological responses (16). After the original description of activation of the PI3K pathway by IFN-α (21), extensive work over time has established important functions for PI3K and its effectors in the generation of IFN responses in various types of normal and malignant cells. These include regulatory effects on induction of apoptosis in neutrophils (40, 41), B lymphocytes (42), and astrocytes (43), as well as promotion of IFN-dependent monocyte adhesion (44). Additionally, there is recent evidence that PI3K can mediate proapoptotic signals in multiple myeloma cells (45). Other recent work has shown that PI3K activity is involved in IFN-γ-dependent regulation of enteric epithelial permeability (46), as well as Fas trafficking and sensitization to apoptosis in vascular smooth muscle cells (47). Interestingly, recent studies have also demonstrated that the function of PI3K is important for nuclear translocation of IRF7 and type I IFN production in response to TLR activation of dendritic cells (48). Despite emerging evidence supporting a key role for PI3K in IFN signaling, the precise mechanisms by which this kinase regulates IFN responses remain to be defined. Previous work in the type II IFN system using pharmacological inhibitors has suggested that activation of the PI3K pathway may be required for IFN-γ-dependent gene transcription, possibly by modifying serine phosphorylation of STAT1 (49). Other earlier studies have also shown that the activation of this pathway by the type I IFN receptor occurs independently of activation of STAT pathways (50), and that it is mediated by the engagement of IRS proteins IRS1 and

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**FIGURE 5.** The function of PI3K is essential for type I and II IFN-dependent gene transcription. A and B, p85α+/+β+/+ and p85α−/−β−/− MEFs were transfected with a β-galactosidase expression vector and either ISRE (A) or 8× GAS (B) luciferase plasmids. Forty-eight hours after transfection, triplicate cultures were either left untreated or treated with IFN-α (A) or IFN-γ (B) for 6 h, and luciferase reporter assays were conducted. The data are expressed as relative luciferase units for each condition, normalized for β-galactosidase activity. Data represent means ± SE values of two independent experiments for A and B, C, p85α+/+β+/+ and p85α−/−β−/− MEFs were incubated for 6 h at 37°C in the absence or presence of mouse IFN-α. Expression of mRNA for the Isg15 gene was evaluated by quantitative RT-PCR (TaqMan). GAPDH was used for normalization. Data are expressed as fold increase over untreated samples and represent means ± SE of four experiments. D, p85α+/+β+/+ and p85α−/−β−/− MEFs were incubated for 6 h at 37°C in the absence or presence of mouse IFN-γ. Expression of mRNA for the Cxcl10 gene was evaluated by quantitative RT-PCR (TaqMan). GAPDH was used for normalization. Data are expressed as fold increase over IFN-γ-untreated samples and represent means ± SE of four experiments.

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**Requirement of PI3K for IFN-inducible mRNA translation and antiviral responses**

In subsequent experiments, we sought to directly determine whether mRNA translation for ISGs is defective in cells with targeted disruption of the p85α and p85β genes. For this purpose, polysomes from mouse IFN-α-treated p85α+/+β+/+ and p85α−/−β−/− MEFs were isolated. The polysomal profiles from wild-type or p85αβ knockout cells, before and after IFN-α treatment, are shown in Fig. 6A. There was an abundance of Isg15 mRNA in

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**Assessed in p85α+/+β+/+ and p85α−/−β−/− MEFs using real-time RT-PCR, there was a significant defect in such transcriptional activation in the knockout cells (Fig. 5, C and D).**

**Discussion**

Beyond the classical Jak-STAT pathways, IFNs activate several other signaling cascades in normal and malignant cells. Such pathways either complement the function of Jak-STAT pathways or mediate IFN-dependent biological responses independently of them (4, 7, 38, 39). Among the alternate pathways activated by IFNs, the PI3K pathway appears to play a prominent role in the generation of biological responses (16). After the original description of activation of the PI3K pathway by IFN-α (21), extensive work over time has established important functions for PI3K and its effectors in the generation of IFN responses in various types of normal and malignant cells. These include regulatory effects on induction of apoptosis in neutrophils (40, 41), B lymphocytes (42), and astrocytes (43), as well as promotion of IFN-dependent monocyte adhesion (44). Additionally, there is recent evidence that PI3K can mediate proapoptotic signals in multiple myeloma cells (45). Other recent work has shown that PI3K activity is involved in IFN-γ-dependent regulation of enteric epithelial permeability (46), as well as Fas trafficking and sensitization to apoptosis in vascular smooth muscle cells (47). Interestingly, recent studies have also demonstrated that the function of PI3K is important for nuclear translocation of IRF7 and type I IFN production in response to TLR activation of dendritic cells (48). Despite emerging evidence supporting a key role for PI3K in IFN signaling, the precise mechanisms by which this kinase regulates IFN responses remain to be defined. Previous work in the type II IFN system using pharmacological inhibitors has suggested that activation of the PI3K pathway may be required for IFN-γ-dependent gene transcription, possibly by modifying serine phosphorylation of STAT1 (49). Other earlier studies have also shown that the activation of this pathway by the type I IFN receptor occurs independently of activation of STAT pathways (50), and that it is mediated by the engagement of IRS proteins IRS1 and

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IRS2 (21–23). Recent evidence has shown that this pathway regulates phosphorylation/activation of the p70 S6 kinase and down-stream phosphorylation of the S6 ribosomal protein in response to both type I and II IFNs (24, 25), raising the possibility that it regulates pathways that control initiation of mRNA translation. However, any potential regulatory effects of PI3K on IFN-dependent mRNA translation had not been directly established, while the potential sequence of signaling events leading to such responses have not been clarified.

In the present study we provide direct evidence that activation of PI3K by the type I and II IFN receptors plays critical and essential roles in the regulation of mRNA translation by IFNs. In studies using knockout cells for both the p85α and p85β isoforms of the p85 regulatory subunit of PI3K, we found that the IFN-α- or IFN-γ engagement of PI3K is essential for type II IFN-dependent mRNA translation of the Cxcl10 gene.

FIGURE 6. Activation of PI3K is essential for type I IFN-regulated mRNA translation of the Isg15 and Ifr7 genes. A, p85α−/−β−/− and p85α−/−β+/− MEFs were either left untreated or treated with mouse IFN-α for 40 h. Cell lysates were separated on 10–50% sucrose gradient, and OD at 254 nm was recorded. The OD at 254 nm is shown as a function of gradient depth for each treatment. B–D, Polysomal fractions were collected as indicated in A and RNA was isolated. Subsequently, quantitative real-time RT-PCR assays to determine Isg15 (B and C) or Ifr7 (D) mRNA expression in polysomal fractions was conducted using GAPDH (B and D) or tubulin (C) for normalization. Data are expressed as fold increase over IFN-α-untreated samples and represent means ± SE for three independent experiments.

FIGURE 7. Engagement of PI3K is essential for type II IFN-dependent mRNA translation of the Cxcl10 gene. A, p85α−/−β−/− and p85α−/−β+/− MEFs were either left untreated or treated with mIFN-γ for 40 h. Cell lysates were separated on 10–50% sucrose gradient and OD at 254 nm was recorded. The OD at 254 nm is shown as a function of gradient depth for each treatment. B and C, Polysomal fractions were collected as indicated in A and RNA was isolated. Subsequently, quantitative real-time RT-PCR assays to determine Cxcl10 mRNA expression in polysomal fractions was conducted, using GAPDH (B) or tubulin (C) for normalization. Data are expressed as fold increase over IFN-γ-untreated samples and represent means ± SE for two independent experiments.
targeted disruption of the genes for the p85α and 85β regulatory subunits of PI3K. p85α+/−/β−/− and p85α−/−/β−/− MEFs were incubated in triplicate with the indicated doses of mouse IFN-α. The cells were subsequently challenged with EMCV, and cytopathic effects (CPE) were quantified. Data are expressed as percentage protection from CPE of EMCV.

inducible phosphorylation/activation of Akt requires upstream activation of PI3K. Similar to results from recent studies using Akt1/ Akt2 double-knockout cells, in which we demonstrated that Akt activation is essential for the IFN activation of mTOR and its effectors (28), the present findings support a mechanism by which PI3K may control mTOR activation and mRNA translation in response to IFNs. We found that expression of key IFN-inducible proteins that regulate the biological effects of IFNs, such as ISG15 and CXCL10, are defective in the absence of the regulatory subunits of PI3K. Importantly, we were able to directly demonstrate that mRNA translation, as assessed in polysomal mRNA fractions, for the Isg15, Ifi77, and Cxcl10 genes is defective in cells lacking p85α and p85β. Thus, there is an essential requirement for PI3K activity in the initiation of mRNA translation by both type I and II IFNs.

Our data also demonstrated that there is defective transcription of IFN-stimulated genes in PI3K knockout cells, as shown both by luciferase reporter assays and in real-time RT-PCR experiments directly examining transcription of the Isg15 and Cxcl10 genes. Interestingly, although the effects of PI3K on IFN-dependent mRNA translation are mediated by Akt activation and downstream engagement of mTOR, the effects on transcription apparently involve an Akt-independent mechanism. This is shown by our previous studies that have demonstrated that IFN-α- or IFN-γ-dependent gene transcription is intact in Akt1/2 double-knockout cells (28). Although the precise downstream signals that mediate such responses remain to be identified, it is possible that they are mediated by activation of different protein kinase C (PKC) isofoms that may regulate gene transcription by distinct mechanisms (51–53), or via engagement of other Akt-independent signals.

Beyond members of the PKC family proteins, another group of kinases known to be regulated in an Akt-independent manner downstream of PI3K includes members of the Tec family of proteins (Btk, Itk, and Rlk) (4). Interestingly, a member of this family of proteins, Bmx, has been previously shown to induce activation of the STAT pathway (54), while recent studies in prostate cells suggest that it is also induced in a PI3K-dependent manner (55). Future studies to determine a potential involvement of Tec kinases and/or other elements in the control of IFN-dependent transcriptional activation will be of interest and may provide additional insights on the regulatory effects of PI3K in IFN signaling.

Our studies also provide direct and definitive evidence establishing that PI3K plays an essential role in the generation of the antiviral effects of IFN-α, as shown by the definitive induction of antiviral responses against EMCV in p85α/β double-knockout cells. Interestingly, another study in which pharmacological inhibitors were used in multiple myeloma cells suggested that PI3K regulates transcription of a subset of IFN-α-stimulated genes that may be involved in the induction of apoptosis (56), although this needs to be confirmed in studies involving genetic approaches. Collectively, our work establishes that PI3K plays dual regulatory roles in IFN signaling, controlling both transcription and mRNA translation of ISGs, and that such function is essential for the induction of the antiviral effects of IFNs in vitro and, possibly, in vivo. Interestingly, it is well established that in response to growth factors and other mitogenic signals, the PI3′-kinase pathway mediates cell proliferative and/or antiapoptotic responses (20, 57). The precise mechanisms that determine the specificity of signals during engagement of this pathway by IFNs remain to be established. It is possible that IFNs compete with growth factors for the use of the same signaling elements required for initiation of cap-dependent translation (i.e., inactivation of 4E-BP1 and subsequent activation of the eIF4E complex) downstream of activation of the PI3K/Akt pathway. It is also likely that initiation of IFN-dependent gene transcription for specific ISGs and the resulting influx of messages for mRNA translation, accompanied by the timely IFN-dependent activation of the mTOR pathway, results in mRNA translation of specific gene products whose transcription is regulated by IFNs. Our studies provide some evidence for the existence of such a mechanism, but additional work will be required to definitively establish the validity of such a hypothesis.

Disclosures

The authors have no financial conflicts of interest.

References