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Interferon γ (IFNγ) Signaling via Mechanistic Target of Rapamycin Complex 2 (mTORC2) and Regulatory Effects in the Generation of Type II Interferon Biological Responses*

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We provide evidence for a unique pathway engaged by the type II IFN receptor, involving mTORC2/AKT-mediated downstream regulation of mTORC1 and effectors. These events are required for formation of the eukaryotic translation initiation factor 4F complex (eIF4F) and initiation of mRNA translation of type II interferon-stimulated genes. Our studies establish that Rictor is essential for the generation of type II IFN-dependent antiviral and antiproliferative responses and that it controls the generation of type II IFN-suppressive effects on normal and malignant hematopoiesis. Together, our findings establish a central role for mTORC2 in IFNγ signaling and type II IFN responses.

IFNs are cytokines that exhibit antiviral, immunomodulatory, growth-inhibitory, and cytotoxic properties (1–12). The critical roles of these cytokines in the innate immune system have provoked clinical interest and extensive studies to explore their therapeutic potential. These studies, spanning several decades, have definitively established their utility in the treatment of viral syndromes, many malignancies, and some autoimmune disorders (1–12).

IFNγ, the sole type II IFN, binds to the IFNGR1 and IFNGR2 subunits of the type II IFN receptor with high affinity and activates the Janus kinases Jak1 and Jak2, leading to engagement of Jak-Stat pathways and transcriptional activation of IFNγ-regulated genes (13–16). Activation of the Jak-Stat pathway is critical for the IFNγ transcriptional control of IFN-stimulated genes (ISGs)[3] and, subsequently, for the generation of IFNγ-induced biological responses (13–16). Beyond the classical Jak-Stat pathways, several other signaling pathways have been shown to be activated by the type II IFN receptor, and their function appears to be critical for IFNγ responses. These include PKC (17), MAP kinase (18, 19), and Mnk kinase cascades (20). There is evidence that the AKT/mTOR pathway is engaged in IFNγ signaling, controlling the initiation of mRNA translation for ISGs (21, 22). However, the precise contribution of different mTOR complexes in this process and the sequence of events leading to ISG mRNA translation remain to be determined.

The mTOR kinase forms the catalytic core of two known complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) (24–35). mTORC1 is a protein complex consisting of mTOR, mammalian lethal with Sec 13 protein 8/G-protein β-protein subunit like (mLST8/GβL), rapamycin-sensitive companion of mTOR (Raptor), Akt/PKB substrate 40 kDa (Pras40), and DEP domain-containing mTOR-interacting protein (Deptor) (24, 25). mTORC1 is known as a key regulator of pathways involved in the initiation of mRNA translation and is inhibited by allosteric inhibitors such as rapamycin, everolimus, temsirolimus, and other rapalogs (24, 25). mTORC2 is comprised of mTOR, mLST8, rapamycin-insensitive companion of mTOR (Rictor), mammalian stress-activated protein kinase interacting protein 1 (Sin1), protein observed with rictor 1/2 (protor 1/2), and deptor (26–32). Although the two mTOR complexes have different effectors and cellular functions, they are both considered important targets for the development of new anticancer agents because they are key promoters of malignant cell growth and survival (24, 29, 31–35). In fact, there is evidence that dual inhibitors of mTORC1 and mTORC2

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[3] The abbreviations used are: ISG, IFN-stimulated gene; mTOR, mechanistic target of rapamycin; MEF, mouse embryonic fibroblast; EMCV, encephalomyocarditis virus; CPE, cytopathic effect.

exhibit more efficient anti-leukemic effects both *in vitro* and *in vivo* compared with specific mTORC1 inhibition (36).

In this study, we examined the engagement of mTORC2 in type II IFN signaling and its role in the generation of IFNγ responses. Our studies demonstrate that mTORC2 is engaged during activation of the type II IFN receptor and exhibits unique functions in IFNγ signaling and that this signaling is essential for mRNA translation of type II ISGs. Importantly, mTORC2 is required for the generation of IFNγ responses, including antiviral effects and effects on normal and malignant hematopoiesis.

Experimental Procedures

Cell Lines and Reagents—Immortalized mouse embryonic fibroblasts (MEFs) were grown in DMEM supplemented with 10% FBS and antibiotics. Immortalized Rictor$^{+/+}$ (rictor$^{Ex3cond/w}$) and Rictor$^{-/-}$ (rictor$^{Ex3del/Ex3del}$) MEFs were provided by Dr. Mark Magnuson (31). Normal CD34^{+} cells were from Stemcell Technologies (Vancouver, Canada). U937 cells were grown in RPMI 1640 medium supplemented with 10% FBS and antibiotics. Phospho-specific antibodies against mTOR, AKT, p70S6K, 4E-BP1, and eIF4B and antibodies against mTOR, AKT, p70S6K, 4E-BP1, and eIF4B were from Cell Signaling Technology (Boston, MA). Phospho-specific antibody against PDCD4 was purchased from Abcam (Cambridge, MA). An antibody against PDCD4 was purchased from Rockland (Gilbertsville, PA). An anti-Rictor antibody was from Bethyl Laboratories (Montgomery, TX). Antibody against GAPDH was from Chemicon, Millipore (Billerica, MA). An antibody against CXCL10 (IP10) was from Abcam, and an anti-IRF9 antibody was from Proteintech Group, Inc. (Chicago, IL). Recombinant human and mouse IFNγ were from Life Technologies.

Cell Lysis and Immunoblotting—Immortalized MEFs were starved overnight in DMEM containing 0.5% FBS and were then treated with mouse IFNγ in DMEM containing 0.5% FBS. U937 cells were transfected with control siRNA or Rictor siRNA, starved overnight in RPMI containing 0.5% FBS, and treated with human IFNγ in RPMI containing 0.5% FBS. Following treatment, cells were washed with PBS and lysed in phosphorylation lysis buffer containing protease and phosphatase inhibitors, as in previous studies (21–22). The lysates were resolved by SDS-PAGE, processed for immunoblotting, and analyzed by enhanced chemiluminescence as in previous studies (21–22).

Cap Binding Assays—These studies were performed as described previously (37, 38). Briefly, Rictor$^{+/+}$ and Rictor$^{-/-}$ MEFs were incubated for 24 h in serum-free medium and then treated with mouse IFNγ for the indicated times. Cell lysates were incubated for 24 h with a dinucleotide mRNA 5′ cap analog (m7GpppG) labeled with biotin attached to ribose of the second dinucleotide (39). After 4-h incubation with streptavidin beads and extensive washing with phosphorylation lysis buffer, the retained proteins were eluted by boiling, resolved by SDS-PAGE, transferred onto Immobilon-P membranes (Millipore), and probed with the indicated antibodies.

Hematopoietic Progenitor Assays—Malignant leukemic progenitor (CFU-L) colony formation in methylcellulose from U937 cells transfected with control siRNA or Rictor siRNA was performed essentially as described previously (40, 41). Normal hematopoietic progenitor colony formation for late erythroid progenitors (BFU-E) or myeloid progenitors (CFU-GM) from normal CD34^{+} bone marrow-derived cells transfected with control siRNA or Rictor siRNA was determined in clonogenic assays in methylcellulose as in our previous studies (41).

Polysomal Isolation—Immortalized Rictor$^{+/+}$ and Rictor$^{-/-}$ MEFs were either left untreated or treated with 1500 IU/ml of mouse IFNγ for 24 h in DMEM supplemented with 0.5% FBS. Cells were washed twice with Dulbecco's PBS with 100 μg/ml cycloheximide and then lysed in lysis buffer (0.5% Triton X-100, 0.5% sodium deoxycholate, 5 mM Tris (pH 7.5), 2.5 mM $MgCl_2$, 1.5 mM KCl, 100 μg/ml cycloheximide, 2 mM DTT, protease inhibitor, and 1 unit/μl RNase inhibitor). Lysates were then centrifuged at 12,000 × *g* for 5 min at 4 °C, and supernatants were collected and snap-frozen in liquid nitrogen. To isolate ribosomal fractions, absorbance at 260 nm was measured for each of the supernatants, and equal amounts of cell lysates of each sample were layered on a sucrose gradient of 5–50%, prepared by using the BioComp Gradient Master 108 (Biocomp Instruments, Fredericton, Canada) according to the instructions of the manufacturer. Samples were centrifuged at 4 °C for 110 min at 35,000 rpm in a Beckman SW41-Ti rotor. The absorbance was measured at 254 nm continuously by a density gradient fractionation system (Brandel, Gaithersburg, MD) with the following settings: pump speed, 0.80 ml/min; fraction size, 10 drops/fraction; chart speed, 300 cm/h; sensitivity, 1; peak separator, off; noise filter, 0.5 s. Assignments of the 40S, 60S, and 80S peaks and polysomes were made on the basis of the absorbance profile. RNA from polysomal fractions was isolated using an RNA All-Prep kit from Qiagen, and 1 μg of polysomal RNA was reverse-transcribed using oligo(dT) primers (Life Technologies) and the Omniscript RT kit (Qiagen) as described previously (22, 23, 37, 38).

Quantitative RT-PCR—Serum-starved Rictor$^{+/+}$ and Rictor$^{-/-}$ MEFs were starved overnight in DMEM containing 0.5% FBS and were then treated for 6 h with 2.5 × 10^3 IU/ml mouse IFNγ. Total RNA was isolated using the RNeasy mini kit (Qiagen) according to the instructions of the manufacturer. 2 μg of total cellular mRNA was reverse-transcribed into cDNA using the Omniscript RT kit (Qiagen) and oligo(dT)12–18 primers (Life Technologies). Real-time PCR was carried out using an ABI7500 sequence detection system (Applied Biosystems) using commercially available 6-fluorescein amidite-labeled probes and primers (Applied Biosystems) to determine mRNA expression of *Cxcl10*, *Irf9*, and *Gapdh*. *Gapdh* was used for normalization as described in our previous studies (22, 23, 37, 38, 41).

Antiviral Assays—The antiviral effects of mouse IFNγ on immortalized Rictor$^{+/+}$ and Rictor$^{-/-}$ MEFs infected with encephalomyocarditis virus (EMCV) were determined as described in our previous studies (22, 23, 40).

Statistical Analyses—Statistical significance was analyzed by Student's *t* test. Differences were considered statistically significant when *p* values were less than 0.05.

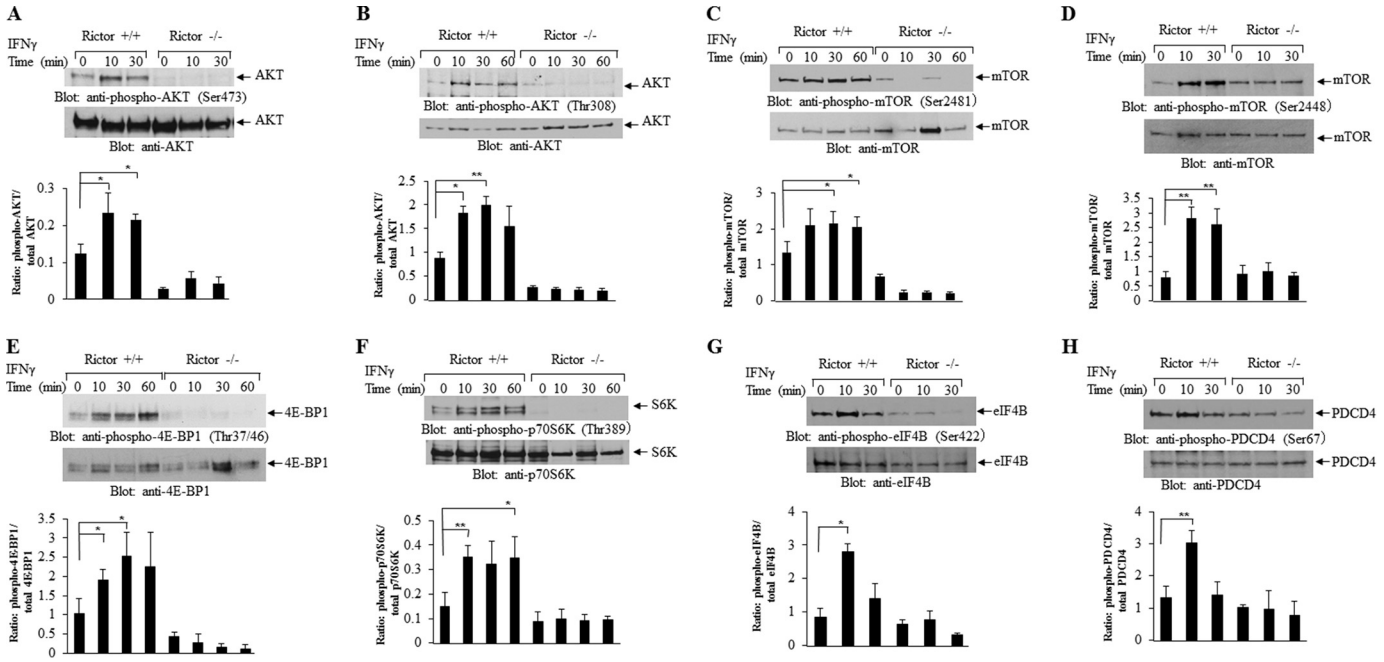

FIGURE 1. **IFNγ-dependent activation of mTORC2 and Rictor-dependent regulatory signals in type II IFN signaling.** *A–H*, serum-starved Rictor$^{+/+}$ and Rictor$^{-/-}$ MEFs were treated with mouse IFNγ for the indicated times. *Top panels*, cell lysates were prepared, and equal amounts of protein were resolved by SDS-PAGE and then subjected to immunoblot analyses with anti-Ser(P)-473 AKT (*A*), anti-Thr(P)-308 AKT (*B*), anti-Ser(P)-2481 mTOR (*C*), anti-Ser(P)-2448 mTOR (*D*), anti-Thr(P)-37/46 4E-BP1 (*E*), anti-Thr(P)-389 p70S6K (*F*), anti-Ser(P)-422 eIF4B (*G*), and anti-Ser(P)-67 PDCD4 antibodies (*H*). The blots in the respective *top panels* were stripped and probed with anti-AKT (*A* and *B*), anti-mTOR (*C* and *D*), anti-4E-BP1 (*E*), p70S6K (*F*), eIF4B (*G*), and PDCD4 antibodies (*H*). *Bottom panels*, bands from three (*A–F*) or two (*G* and *H*) independent experiments (including the blots shown) were quantified by densitometry. Data are expressed as mean of ratios of phosphoprotein over respective total protein ± S.E. for each experimental condition. Statistical analyses were performed using Student's *t* test as indicated. *, $p < 0.05$; **, $p < 0.01$.

Results

Previous studies have provided evidence for AKT activation in type II IFN signaling (21, 22), but the precise mechanisms of its engagement remained undefined. Accordingly, we undertook studies to determine whether mTORC2 is engaged by the type II IFN receptor and regulates IFNγ-dependent AKT activation. In initial studies, we compared type II IFN-dependent phosphorylation of AKT in wild-type cells and Rictor knockout MEFs. IFNγ treatment resulted in phosphorylation of AKT on Ser-473 and Thr-308 in wild-type MEFs but not in Rictor knockout MEFs (Fig. 1, *A* and *B*, respectively), establishing a requirement for Rictor in the process. These findings are in agreement with previous studies in other systems demonstrating that mTORC2 activation is required for phosphorylation of AKT on Ser-473 and that this phosphorylation is required for enabling PDK1 to phosphorylate AKT at Thr-308 (27). Similarly, phosphorylation of mTOR on Ser-2481, a site whose phosphorylation correlates with mTORC2 activity (42, 43), was defective in Rictor knockout cells (Fig. 1*C*). Taken together, these studies suggest that IFNγ induces activation of mTORC2 in a Rictor-dependent manner. Notably, phosphorylation of mTOR on Ser-2448 was also Rictor-dependent (Fig. 1*D*). Earlier evidence suggested that the mTOR phosphorylation sites Ser-2481 and Ser-2448 may correlate with mTORC2 and mTORC1 catalytic activity, respectively (44). The defective type II IFN-dependent phosphorylation of mTOR on Ser-2448, viewed together with our previous studies implicating AKT upstream of mTORC1 in IFNγ signaling (22, 42), led us to further studies to examine the possibility that engagement of mTORC2 lies upstream of mTORC1 in the type II IFN system. IFNγ-induced phosphorylation of 4E-BP1 on Thr-37/46 (Fig. 1*E*) and S6K on Thr-389 (Fig. 1*F*) was reduced in Rictor$^{-/-}$ MEFs (Fig. 1, *E* and *F*). Similarly, IFNγ-dependent phosphorylation of the downstream effector of S6K, eIF4B, (23), was decreased in Rictor$^{-/-}$ MEFs (Fig. 1*G*). Our previous work has demonstrated that phosphorylation of the translational repressor programmed cell death 4 (PDCD4) protein on Ser-67 also occurs in a S6K-dependent manner (37) in the type I IFN system. IFNγ treatment resulted in induction of phosphorylation of PDCD4 on Ser-67 in Rictor$^{+/+}$ MEFs, but this phosphorylation was defective in Rictor$^{-/-}$ MEFs (Fig. 1*H*).

To further establish the role of Rictor in the regulation of type II IFN-dependent mTOR activity, we performed studies in U937 hematopoietic cells, employing transient knockdown of Rictor by specific siRNA-mediated targeting. As shown in Fig. 2, IFNγ-dependent phosphorylation of AKT (Fig. 2, *A* and *B*) and S6K (Fig. 2*C*) were impaired substantially in cells with Rictor knockdown, consistent with the findings in Rictor$^{-/-}$ MEFs.

We next examined whether the function of Rictor is required for expression of IFNγ-inducible proteins. We determined the expression of IFNγ-induced protein CXCL10 (IP10), a chemokine that has diverse roles in infectious diseases, induction of apoptosis, and cell growth inhibition (45). A strong induction of CXCL10 expression was seen in Rictor$^{+/+}$ MEFs upon IFNγ treatment, but this induction was defective in Rictor$^{-/-}$ MEFs (Fig. 3*A*). Consistent with these findings, CXCL10 expression was also reduced in U937 cells in which Rictor was knocked

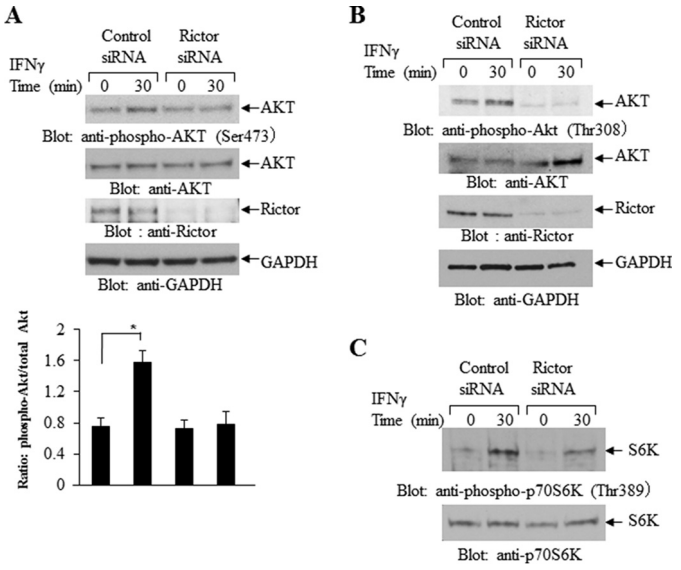

FIGURE 2. **Rictor requirement for type II IFN-inducible, mTOR-mediated signaling events.** *A* and *B*, serum-starved U937 cells transfected with control siRNA or Rictor siRNA were treated with human IFNγ for the indicated times. Cell lysates were prepared, and proteins were resolved by SDS-PAGE and then processed for immunoblotting with anti-Ser(P)-473 AKT (*A*) and anti-Thr(P)-308 AKT, anti-Rictor, and anti-GAPDH antibodies (*B*) as indicated. The blots were stripped and probed with an anti-AKT antibody. Bands from three independent experiments were quantified by densitometry. Data are expressed as mean ± S.E. of ratios of phospho-AKT over total AKT for each experimental condition. Statistical analysis was performed using Student's *t* test as indicated. *, $p < 0.05$. *C*, serum-starved U937 cells transfected with control siRNA or Rictor siRNA were treated with human IFNγ for the indicated times. Lysates were analyzed by SDS-PAGE and immunoblotted with an anti-Thr(P)-389 p70S6K antibody. The blot was then stripped and reprobed with an antibody against p70S6K.

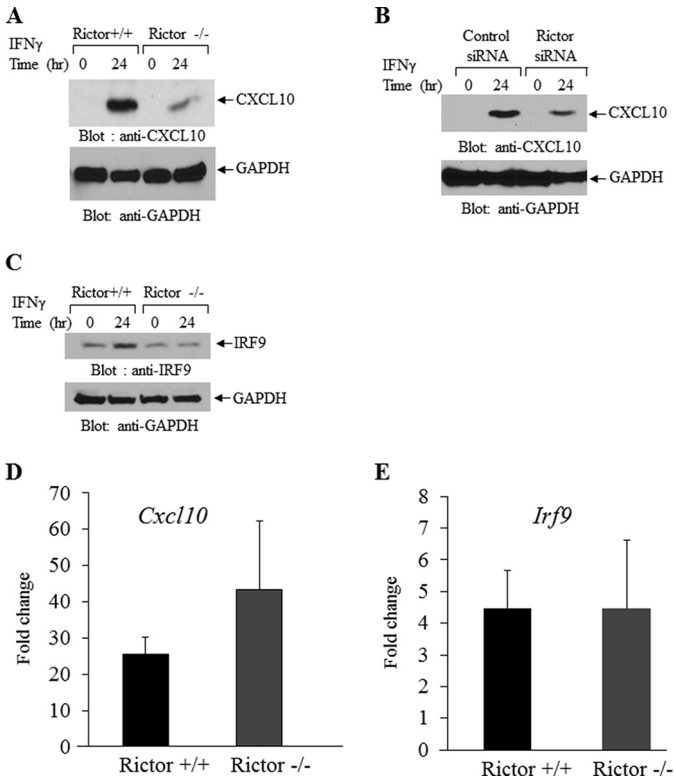

FIGURE 3. **Type II IFN-induced CXCL10 and IRF9 expression is Rictor-dependent.** *A*, serum-starved Rictor$^{+/+}$ and Rictor$^{-/-}$ MEFs were treated with mouse IFNγ as indicated. Cell lysates were prepared, proteins were resolved by SDS-PAGE, and immunoblots were probed with anti-CXCL10 antibody. *B*, serum-starved U937 cells transfected with control siRNA or Rictor siRNA were treated with human IFNγ for the indicated times. Cell lysates were prepared, and proteins were resolved by SDS-PAGE and then processed for immunoblotting with an anti-CXCL10 antibody. *C*, serum-starved Rictor$^{+/+}$ and Rictor$^{-/-}$ MEFs were treated with mouse IFNγ as indicated. Cell lysates were prepared, proteins were resolved by SDS-PAGE, and immunoblots were probed with an anti-IRF9 antibody. The blots in the respective *top panels* (*A–C*) were probed with an anti-GAPDH antibody, as indicated, to control for protein loading. *D* and *E*, serum-starved Rictor$^{+/+}$ or Rictor$^{-/-}$ MEFs were left untreated or treated with mouse IFNγ for 6 h, and total RNA was isolated. The mRNA expression of *Cxcl10* (*D*) and *Irf9* (*E*) was evaluated by quantitative real-time PCR, and *Gapdh* was used for normalization. Data are expressed as -fold change over control untreated cells, and *error bars* represent mean ± S.E. of three independent experiments.

down using specific siRNAs (Fig. 3*B*). We also examined whether Rictor is required for the expression of IRF9, which is required for the transcription of type I IFN-stimulated genes (46). As expected, IFNγ treatment resulted in induction of expression of IRF9 in Rictor$^{+/+}$ MEFs (Fig. 3*C*). However, this induction was defective in Rictor$^{-/-}$ MEFs (Fig. 3*C*).

Next we conducted studies to define the mechanisms of regulation of type II IFN-inducible protein expression by Rictor. RNA from Rictor$^{+/+}$ and Rictor$^{-/-}$ MEFs treated with IFNγ was examined by quantitative real-time RT-PCR to compare the -fold increase in *Cxcl10* and *Irf9* mRNA in the presence or absence of Rictor. Lack of Rictor expression did not reduce IFNγ-dependent *Cxcl10* and *Irf9* total mRNA expression (Fig. 3, *D* and *E*), suggesting that there are no type II IFN transcriptional defects in MEFs lacking Rictor. This led us to consider the possibility that defective expression of type II IFN-regulated proteins in the absence of Rictor reflects impaired ISG mRNA translation. Using the 5′ cap binding assay (38), we undertook studies to determine whether the function of Rictor in IFNγ signaling ultimately controls downstream assembly of the translation initiation factor complex. As shown in Fig. 4*A*, IFNγ treatment resulted in enhanced binding of eIF4G and eIF4A to the 5′ cap structure in Rictor$^{+/+}$ MEFs but not in Rictor$^{-/-}$ MEFs. Notably, IFNγ treatment resulted in dissociation of 4E-BP1 from eIF4E in the 5′ cap translation initiation complex in Rictor$^{+/+}$ MEFs but not in Rictor$^{-/-}$ MEFs (Fig. 4*A*). To determine whether impaired IFNγ-inducible engagement of mTOR effectors in Rictor$^{-/-}$ MEFs accounts for defective mRNA translation of type II IFN-dependent ISGs, we examined and compared *Cxcl10* and *Irf9* mRNA expression in polysome fractions from Rictor$^{+/+}$ and Rictor$^{-/-}$ MEFs following treatment with IFNγ (Fig. 4*B*). Quantitative RT-PCR analyses were used to measure the amount of *Cxcl10* and *Irf9* mRNA levels in polysomes. Both *Cxcl10* (Fig. 4*C*) and *Irf9* (Fig. 4*D*) polysomal mRNA levels were decreased in Rictor$^{-/-}$ cells compared with Rictor$^{+/+}$ MEFs after IFNγ treatment, consistent with defects in mRNA translation of ISGs in the absence of Rictor.

In parallel, we examined the role of Rictor in the generation of the anti-leukemic activity of IFNγ. We evaluated the effects of Rictor knockdown in the generation of the suppressive effects of IFNγ on leukemic progenitor colony formation. As expected, IFNγ treatment suppressed CFU-blast (CFU-L) colony formation from U937 cells transfected with control siRNA (Fig. 5*A*). However, this IFNγ inhibition was partially reversed by Rictor knockdown (Fig. 5*A*), suggesting that Rictor is an essential mediator for induction of the anti-leukemic properties of IFNγ. We also examined the functional relevance of Ric-

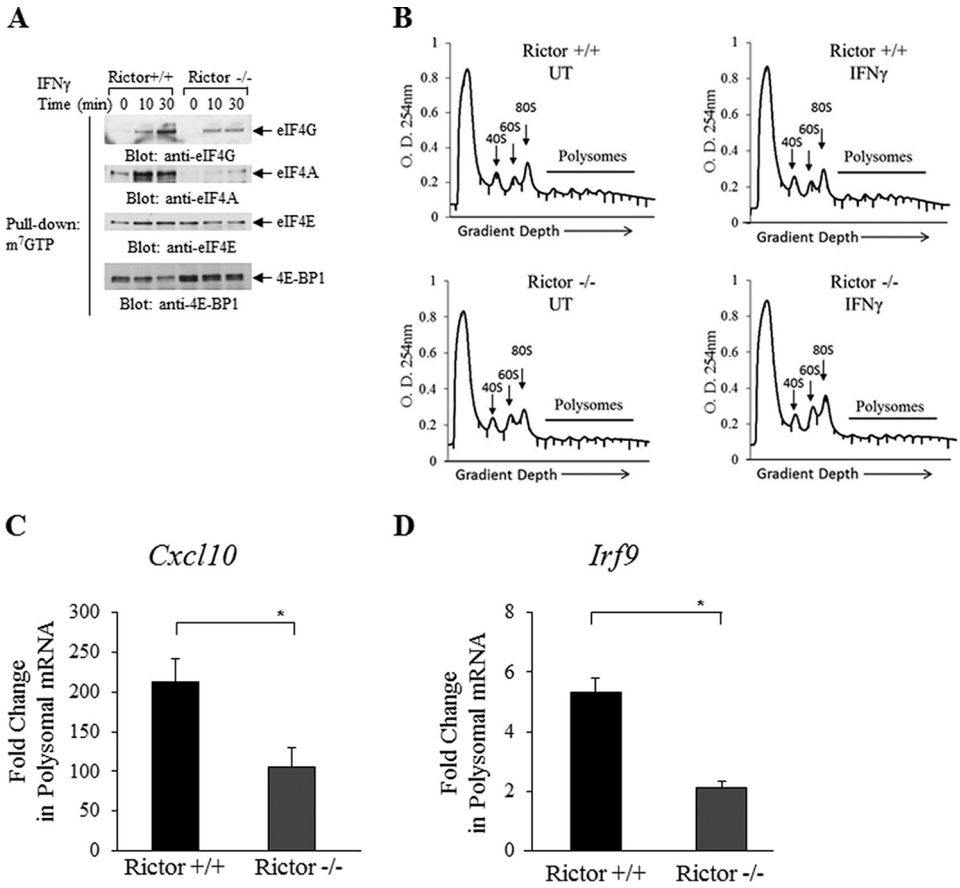

FIGURE 4. **Essential role of Rictor in mRNA translation of type II ISGs.** *A*, serum-starved Rictor$^{+/+}$ and Rictor$^{-/-}$ MEFs were either left untreated or treated with mouse IFNγ. Cell lysates were bound to the biotin labeled cap analog, m^{7}GTP, and to streptavidin beads (39). After extensive washing, bound proteins were resolved by SDS-PAGE and immunoblotted with antibodies against eIF4G, eIF4A, eIF4E, and 4E-BP1. The cell lysates used for this experiment were from the same experiment shown in Fig. 1*D*. *B*, Rictor$^{+/+}$ and Rictor$^{-/-}$ MEFs were either left untreated or treated with mouse IFNγ in DMEM containing 0.5% FBS for 24 h. Cell lysates were layered on 5–50% sucrose gradients and subjected to density gradient centrifugation, and then fractions were collected by continuous monitoring of absorbance at 254 nm. Absorbance at 254 nm is shown as a function of gradient depth, and the 40S, 60S, and 80S peaks and polysomal fractions are indicated. *C* and *D*, polysomal fractions were pooled, and RNA was isolated. Subsequently, quantitative real-time PCR was carried out to determine *CxCl10* (*C*) and *Irf9* (*D*) mRNA expression in polysomal fractions, using *Gapdh* for normalization. Data are expressed as -fold change over control untreated cells, and *error bars* represent mean ± S.E. of five independent experiments, including the ones shown in *B*. Statistical analyses were performed using Student's *t* test. *, $p < 0.05$.

tor in mediating the suppressive effects of IFNγ on normal hematopoiesis. Normal human CD34$^+$ bone marrow cells transfected with control siRNA or siRNA specifically targeting human Rictor were treated with IFNγ, and myeloid (CFU-GM) or erythroid (BFU-E) colony formation was assessed in clonogenic assays in methylcellulose. As shown in Fig. 5*B*, treatment with IFNγ resulted in suppression of hematopoietic progenitor colony formation, but these effects were partially reversed by Rictor knockdown (Fig. 5*B*).

Finally, we examined the potential involvement of Rictor in the generation of the antiviral effects of IFNγ. The antiviral activity of mouse IFNγ against EMCV infection was examined in Rictor$^{-/-}$ MEFs and compared with the effects seen in parental MEFs. Parental MEFs were responsive to the antiviral effects of IFNγ, as reflected by protection from the cytopathic effects (CPEs) of EMCV (Fig. 5*C*). However, the antiviral effects of IFNγ were reduced in Rictor$^{-/-}$ MEFs (Fig. 5*C*), consistent with Rictor having an important role in the generation of IFNγ-induced antiviral responses. On the other hand, when the effects of rapamycin on IFNγ-dependent antiviral responses were assessed, there were no significant differences between rapamycin-treated and untreated Rictor$^{+/+}$ MEFs (Fig. 5*D*), suggesting that rapamycin-sensitive mTORC1 complexes are not involved in the IFNγ-dependent antiviral response.

Discussion

IFNγ has minimal homology with type I IFNs and binds to a unique cell surface receptor, the type II IFN receptor (1–2). The signaling pathways activated by the type II IFN receptor and the mechanisms accounting for IFNγ responses are of particular interest because of the critical role of IFNγ in immune responses to a variety of insults. Moreover, IFNγ overproduction has been implicated in the pathophysiology of certain diseases, especially bone marrow failure syndromes in humans (12, 47, 48). Therefore, precisely defining the signaling pathways downstream of the type II IFN receptor and elucidating their roles in specific biological responses has important clinical translational implications for the design of antiviral therapies and for the design of targeting approaches in bone marrow failure disorders and syndromes.

Type II IFN signaling includes transcriptional activation of ISGs via Jak-Stat pathways (15, 16). Accumulating evidence has

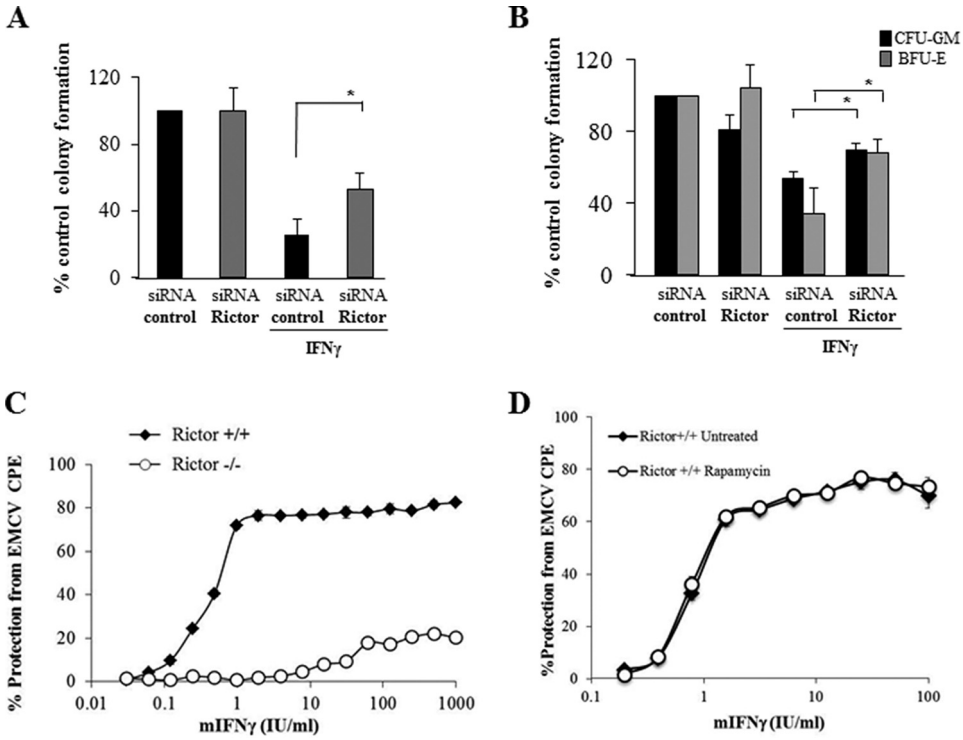

FIGURE 5. **Requirement of intact mTORC2 for the generation of IFNγ-biological effects.** *A*, U937 cells were transfected with either control siRNA or siRNA specifically targeting Rictor, as indicated. The cells were subsequently plated in methylcellulose in the absence or presence of human IFNγ, and leukemic CFU-L colony formation was assessed. Data are expressed as the percentage of colony formation of untreated control siRNA-transfected cells, and *error bars* represent mean ± S.E. of four independent experiments. Statistical analysis was performed using Student's *t* test as indicated. *, $p < 0.05$. *B*, normal human CD34$^+$ bone marrow-derived cells were transfected with control siRNA or Rictor siRNA and incubated in clonogenic assays in methylcellulose in the presence or absence of human IFNγ as indicated. CFU-GM and BFU-E progenitor colonies were scored after 14 days in culture. Data are expressed as the percentage of colony formation of untreated control siRNA-transfected cells, and *error bars* represent mean ± S.E. of three independent experiments. Statistical analyses were performed using Student's *t* test as indicated. *, $p < 0.05$. *C*, Rictor$^{+/+}$ and Rictor$^{-/-}$ MEFs were incubated with the indicated doses of mouse IFNγ and subsequently challenged with EMCV. Virus-induced CPEs were quantified 4 days later. Data are expressed as percent protection from EMCV CPEs and are representative of three independent experiments. Values are mean + S.E. of quadruplicate assays. *D*, Rictor$^{+/+}$ MEFs were pretreated with the indicated doses of mouse IFNγ for 16 h and then challenged with EMCV. In parallel, Rictor$^{+/+}$ MEFs were pretreated for 60 min with rapamycin (20 nM) and then with the indicated doses of mouse IFNγ for 16 h in the continuous presence of rapamycin (20 nM) and subsequently challenged with EMCV. Virus-induced CPEs were quantified 24 h post-infection. Data are expressed as percent protection from EMCV CPEs. Values are mean + S.E. of quadruplicate assays for two independent experiments.

implicated serine kinase pathways in the optimal transcriptional activation of type II IFN genes via regulation of STAT serine phosphorylation (15, 16). More recently, studies have focused on the identification of mechanisms by which IFNs regulate mRNA translation (49). There is accumulating evidence indicating central and essential roles for IFN-activated AKT/mTOR pathways and effector elements in type II IFN-signaling (23, 24, 47, 50). Notably, mTOR signals are well recognized to regulate cap-dependent mRNA translation and protein expression of tumorigenic proteins and play positive roles in cytokine-induced mitogenic signaling pathways and immune signaling networks (24, 29, 32–35).

In this study we provide the first evidence implicating mTORC2 in type II IFN signaling and provide direct evidence establishing that mTORC2 has a critical role in Type II IFN responses. This involves a unique cascade (mTORC2 → AKT → mTORC1 → cap effectors) that is not seen in growth factor or other cytokine signaling pathways (51), with the exception of type I IFNs (40, 52). Our findings establish that IFNγ, a cytokine that exhibits antiproliferative and antiviral effects but has minimal homology to type I IFNs, also engages mTORC2 in a non-classical way. Moreover, our data strongly suggest that IFNγ receptor-elicited specific signals modify mTORC2 in a unique way that results in downstream engagement of mTORC1 and effectors that mediate cap-dependent translation. The uniqueness of the pathway and the lack of similar Rictor/mTORC2-dependent signaling events in mitogenic/neoplastic pathways raise the possibility that type II IFNR-dependent specific utilization of Rictor is ultimately required for the induction of antiproliferative and antiviral responses via control of mRNA translation of specific type II ISGs.

Together, the findings of this study establish that the function of Rictor is required for the generation of IFNγ-induced biological responses, including the suppressive effects of this cytokine in normal hematopoiesis. The specificity of this unique signaling cascade makes it an attractive target for therapeutic approaches aimed at selectively reversing the suppression of hematopoiesis resulting from IFNγ overproduction in hematopoiesis (47, 48). It is conceivable that future identification of IFN-specific signaling elements that drive the mTORC2 → AKT → mTORC1 → cap effector sequence of events will ultimately allow the development of specific therapeutic interventions to target the pathway and selectively disrupt production of ISG products that suppress hematopoiesis. Moreover, a better understanding of the involvement of this pathway in the generation of antiviral responses may provide leads for the design of novel antiviral therapeutic approaches.

Author Contributions—B. K. designed and performed the research and analyzed the data. R. L. R., E. M. K., B. M. K., D. S., A. D. A., S. M., and G. T. B. performed the research and analyzed the data. J. J. and Z. W. developed, produced, and provided key experimental reagents for the study. E. N. F. and L. C. P. conceived and designed the research and analyzed data. B. K., D. S., R. L. R., E. N. F., and L. C. P. contributed to manuscript drafting. All authors reviewed the results and approved the final version of the manuscript.

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