Regulatory effects of mTORC2 complexes in type I IFN signaling and in the generation of IFN responses

Surinder Kaur[a], Antonella Sassano[a], Beata Majchrzak-Kita[b,c], Darren P. Baker[d], Bing Su[e], Eleanor N. Fish[b,c], and Leonidas C. Platanias[a,1]

[a]Robert H. Lurie Comprehensive Cancer Center and Division of Hematology–Oncology, Northwestern University Medical School and Jesse Brown Veterans Affairs Medical Center, Chicago, IL 60611; [b]Division of Cell and Molecular Biology, Toronto General Research Institute, University Health Network, Toronto, ON, Canada M5G 2M9; [c]Department of Immunology, University of Toronto, Toronto, ON, Canada M5G 2M1; [d]Biogen Idec, Cambridge, MA 02142; and [e]Department of Immunology, Yale University School of Medicine, New Haven, CT 06520

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IFNs transduce signals by binding to cell surface receptors and activating cellular pathways and regulatory networks that control transcription of IFN-stimulated genes (ISGs) and mRNA translation, leading to generation of protein products that mediate biological responses. Previous studies have shown that type I IFN receptor-engaged pathways downstream of AKT and mammalian target of rapamycin complex (mTORC) 1 play important roles in mRNA translation of ISGs and the generation of IFN responses, but the roles of mTORC2 complexes in IFN signaling are unknown. We provide evidence that mTORC2 complexes control IFN-induced phosphorylation of AKT on serine 473 and their function is ultimately required for IFN-dependent gene transcription via interferon-stimulated response elements. We also demonstrate that such complexes exhibit regulatory effects on other IFN-dependent mammalian target of rapamycin-mediated signaling events, likely via engagement of the AKT/mTORC1 axis, including IFN-induced phosphorylation of S6 kinase and its effector rpS6, as well as phosphorylation of the translational repressor 4E-binding protein 1. We also show that induction of ISG protein expression and the generation of antiviral responses are defective in Rictor and mLST8-KO cells. Together, our data provide evidence for unique functions of mTORC2 complexes in the induction of type I IFN responses and suggest a critical role for mTORC2-mediated signals in IFN signaling.

The cytokine family of IFNs includes several groups and members with diverse pleiotropic biological functions, including antiviral, immunomodulatory, and growth-inhibitory properties (1, 2). Over the years, IFNs have found various clinical applications and have been used extensively in the treatment of malignancies, viral syndromes, and neurological disorders such as multiple sclerosis (3, 4). IFNs generate their effects on target cells by binding to specific cell surface receptors and activating associated JAK kinases. JAK kinases phosphorylate and activate STAT proteins, which translocate to the nucleus and bind to the promoters of IFN-stimulated genes (ISGs) (2, 5, 6). Such IFN-activated JAK–STAT pathways are critical for the generation of IFN responses, as they control transcription of ISGs, ultimately giving rise to protein products that mediate the biological effects of IFNs (2, 5, 6). Several ancillary pathways are also activated by IFNs (6) and play roles in transcription and/or mRNA translation of ISGs. Among these pathways are the p38 MAP kinase pathway (7, 8), the MAP kinase kinase/ERK/MAPK signal-interacting kinase cascade (9, 10), and the PI3K–AKT–mammalian target of rapamycin (mTOR) signaling pathway (11–16). p38 MAPK-generated signals complement the function of JAK–STAT pathways and are required for optimal transcription of ISGs (8), whereas Mnk- and AKT-mTOR signaling events are required for ISG mRNA translation (14–19).

In previous work, we demonstrated that the mTOR pathway is activated by the type I IFN receptor (IFNR) and have dissected the roles of various components of this signaling cascade in the generation of IFN responses. By using murine embryonic fibroblast (MEF) cells with genetic disruption of various elements of the mTOR pathway, we demonstrated that the translational suppressor 4E-binding protein 1 (4E-BP1) and tuberous sclerosis proteins, TSC1 and TSC2, exert negative regulatory roles in the generation of IFN responses (13). In other studies, we have shown that the AKT kinase is engaged by IFNs and plays an essential regulatory role in mRNA translation of ISGs (14). mTOR exists in two complexes, mTOR complex (mTORC) 1 and mTORC2 (20–22), with mTORC2 regulating phosphorylation of AKT at its serine 473 site (20–23). mTORC1 comprises mTOR, mLST8, Raptor, and Pras40, whereas mTORC2 is composed of mTOR, Rictor, Sin1, and mLST8 (20-27). Although mLST8 is present in mTORC1 and mTORC2 complexes, it has been shown that the phenotype of the mLST8-KO embryos resembles that of the Rictor KO (27). In addition, there is evidence that mLST8 is more important for the assembly of mTORC2 than mTORC1 (27).

In the present study, we used cells with targeted disruption of various components of mTORC2, including Rictor, Sin1, and mLST8, to examine the roles of mTORC2 complexes in the generation of type I IFN responses. Our studies show that mTORC2 plays essential roles in the ultimate expression of ISG products and the generation of IFN biological responses. In contrast to what has been shown for growth factor signaling and prooncogenic responses, S6 kinase (S6K) and rpS6 appear to require upstream engagement of mTORC2, to be regulated and activated downstream of IFN-activated mTORC1. Such unexpected relative specificity raises the possibility of differential functions and activities of mTORC2 in response to cytokines with growth suppressive properties vs. cytokines and growth factors that promote cell proliferation.

Results

Rictor is an essential component of mTORC2 and is required for assembly and function of this complex (22–25, 27–29), whereas mTORC2 phosphorylates serine 473 site in the hydrophobic motif of AKT (22–24, 29). In initial studies, we examined the ability of IFN-α to induce AKT phosphorylation in MEFs with targeted disruption of the *Rictor* gene (30). As expected, there was induction of AKT phosphorylation on Ser-473 in a Type I IFN-dependent manner in WT MEFs (Fig. 1*A*). However, such phosphorylation was defective in Rictor$^{-/-}$ MEFs (Fig. 1*A*), establishing that Rictor is required for IFN-dependent Ser-473 phosphorylation. We also examined the ability of IFN to induce phosphorylation of AKT in MEFs with genetic disruption of two other genes, *Sin1* (31) and *mLST8* (27), the protein products of which are also required for assembly and function of the mTORC2 complex. Type I IFN-induced phosphorylation of AKT on Ser-473 was defective in MEFs with genetic disruption of Sin1 (i.e., Sin1$^{-/-}$; Fig. 1*B*) or mLST8 (i.e., mLST8$^{-/-}$; Fig. 1*C* and Fig. S1*A*). We next examined type I IFN-induced phosphorylation of Pras40 at Thr246, which is an AKT target site

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[1]To whom correspondence should be addressed. E-mail: l-platanias@northwestern.edu.

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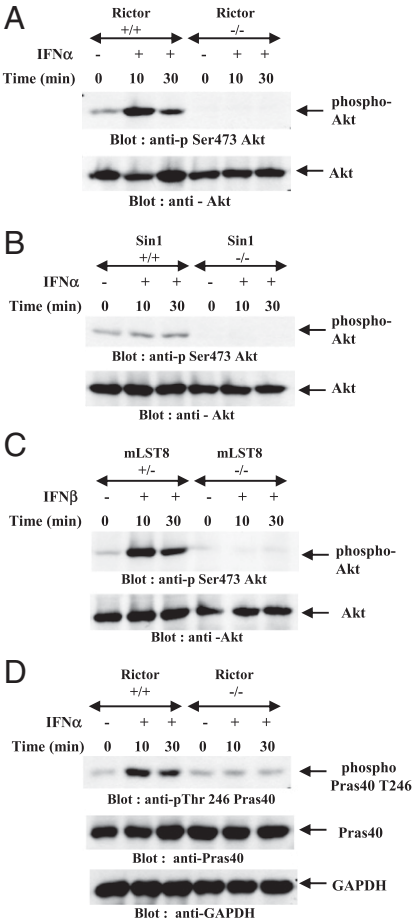

Fig. 1. Type I IFN-induced phosphorylation of AKT is mTORC2-dependent. (*A*) Rictor$^{+/+}$ or Rictor$^{-/-}$ MEFs were treated with mouse IFN-α for the indicated times. Equal protein amounts were subjected to immunoblot analysis with an anti–phospho-Ser-473-AKT antibody. The blot was then stripped and reprobed with an anti-AKT antibody as indicated. (*B*) Sin1$^{+/+}$ or Sin1$^{-/-}$ MEFs were treated with mouse IFN-α for the indicated times. Equal protein amounts were subjected to immunoblot analysis with an anti–phospho-Ser-473-AKT antibody. The blot was then stripped and reprobed with an anti-AKT antibody as indicated. (*C*) mLST8$^{+/-}$ or mLST8$^{-/-}$ MEFs were treated with mouse IFN-β for the indicated times. Equal protein amounts were subjected to immunoblot analysis with an anti–phospho-Ser-473-AKT antibody. The blot was then stripped and reprobed with an anti-AKT antibody as indicated. (*D*) Rictor$^{+/+}$ or Rictor$^{-/-}$ MEFs were treated with mouse IFN-α for the indicated times. Equal protein amounts were subjected to immunoblot analysis with an anti–phospho-Thr246 Pras40 antibody. The same blot was then stripped and reprobed with anti-Pras40 or anti-GAPDH antibodies.

(32). There was strong type I IFN-dependent Pras40 phosphorylation in Rictor$^{+/+}$ MEFs, but not in Rictor$^{-/-}$ MEFs (Fig. 1*D*), further demonstrating the requirement of Rictor for IFN-inducible AKT activity. Similarly, IFN-dependent Pras40 phosphorylation was induced in Sin1$^{+/+}$ and mLST8$^{+/-}$ MEFs, but not in Sin1$^{-/-}$ and mLST8$^{-/-}$ MEFs (Fig. S2). Together, these studies established that mTORC2 complexes are essential for IFN-induced AKT phosphorylation and activation.

As IFN-dependent activation of mTOR and its effectors occurs downstream of AKT (14), we examined the effects of targeted disruption of *Rictor* on IFN-inducible phosphorylation of downstream effectors of mTOR. IFN-α induced phosphorylation of p70 S6K on Thr-389 (Fig. 2*A*) and phosphorylation of its downstream effector rpS6 (Fig. 2*B*) in WT MEFs, but this phosphorylation was substantially decreased in Rictor$^{-/-}$ MEFs (Fig. 2 *A* and *B*, respectively). IFN-α–induced p70S6K phosphorylation was also defective in Sin1$^{-/-}$ MEFs (Fig. 2*C*). Although there were higher basal levels of rpS6 phosphorylation in Sin1$^{-/-}$ MEFs, possibly reflecting compensation by another kinase, IFN-dependent induction of rpS6 phosphorylation was not observed (Fig. 2*D*). Moreover, in studies using mLST8$^{-/-}$ MEFs, we noticed defective type I IFN-dependent phosphorylation of S6K and rpS6 (Fig. 2 *E* and *F* and Fig. S1 *B* and *C*). We also examined the phosphorylation of 4E-BP1 on Thr-37/46 in Rictor$^{+/+}$ and Rictor$^{-/-}$ MEFs. There was an IFN-dependent increase of 4E-BP1 phosphorylation on Thr37/46, Thr70, and Ser65 in Rictor$^{+/+}$ MEFs, but this phosphorylation was defective or very weak in Rictor$^{-/-}$ MEFs (Fig. 2 *G–I*). When similar studies were conducted in Sin1$^{+/+}$ and Sin1$^{-/-}$ MEFs, we found that there was an IFN-inducible increase in 4E-BP1 phosphorylation in parental Sin1$^{+/+}$ MEFs (Fig. 2 *J* and *K*). On the contrary, Sin1$^{-/-}$ MEFs exhibited a high basal level of 4E-BP1 phosphorylation on Thr37/46 and Thr70, which did not change with IFN treatment (Fig. 2 *J* and *K*). Type I IFN-induced phosphorylation of 4E-BP1 on Thr37/46 was also seen in mLST8$^{+/-}$ MEFs, but this induction was defective in mLST8$^{-/-}$ MEFs (Fig. 2*L*).

Previous reports have demonstrated that insulin- or growth factor-induced phosphorylation of S6K is similar in Rictor-KO cells and parental cells that express Rictor (27, 30). Because our data suggested defective IFN-dependent engagement of S6K and rpS6, we compared the induction of phosphorylation of S6K in response to type I IFN treatment, insulin, or serum treatment in Rictor$^{+/+}$ and Rictor$^{-/-}$ MEFs. As shown in Fig. 3*A*, there was induction of Thr389 S6K phosphorylation by IFN-α, insulin, or serum in Rictor$^{+/+}$ MEFs. Insulin and serum induced phosphorylation of S6K in immortalized MEFs with disruption of the *Rictor* gene (30), but this phosphorylation was not seen in response to IFN treatment of Rictor$^{-/-}$ MEFs (Fig. 3*A* and Fig. S3). Similarly, by using MEFs from a different Rictor KO (27), we found that there was very weak S6K phosphorylation in response to type I IFN-treatment, whereas insulin or serum induced S6K phosphorylation (Fig. S4). In addition, insulin or serum induced rpS6 phosphorylation at Ser-235/236 in Rictor$^{+/+}$ and Rictor$^{-/-}$ MEFs, but IFN treatment did not result in rpS6 phosphorylation in Rictor$^{-/-}$ MEFs (Fig. 3*B*). IFN-induced phosphorylation of Pras40 on Thr246 was also diminished compared with insulin and serum in Rictor$^{-/-}$ MEFs (Fig. 3*C* and Fig. S4).

We next examined whether the function of Rictor is required for expression of ISGs. We determined the IFN-inducible expression of ISG15, a protein whose function is critical for the antiviral effects of type I IFNs (33). There was a strong induction of ISG15 expression in Rictor$^{+/+}$ MEF cells treated with IFN-α or IFN-β, but this induction was defective in Rictor$^{-/-}$ MEFs (Fig. 4*A*). We also assessed whether Rictor expression is required for induction of ISG54, a protein product implicated in IFN-inducible antiviral and proapoptotic properties (34, 35). There was strong induction of ISG54 by IFN-α and IFN-β in Rictor$^{+/+}$ MEFs, but this induction was defective in Rictor$^{-/-}$ MEFs (Fig. 4*B*). In parallel studies, we found strong induction of ISG15 and ISG54 expression in Sin1$^{+/+}$ MEFs, but there was much weaker induction in Sin1$^{-/-}$ MEFs (Fig. 4 *C* and *D*). Similar results were obtained when mLST8$^{-/-}$ MEFs were used, in which case a decrease in induction of ISG15 and ISG54 expression by IFNs was observed compared with mLST8$^{+/-}$ cells (Fig. 4 *E* and *F*). Thus, various components of the mTORC2 complex appear to play critical roles in regulating expression of ISG proteins with key functions as mediators of IFN responses. To further establish the role of mTORC2 complexes in IFN-mediated ISG15 and ISG54 protein expression, in a system other than MEF cells, we generated U937 cells with stable knockdown of Rictor by using lentiviral vectors (Fig. 4*G*). In experiments in which control shRNA or Rictor shRNA-infected U937 cells were treated with human IFN-α, and induction of ISG15 and ISG54 was studied, we found decreased expression of ISG15 and ISG54 in cells with stable knockdown of Rictor (Fig. 4 *H* and *I*), consistent with the results of studies that used Rictor$^{-/-}$ MEFs.

In subsequent studies, we sought to determine the mechanisms that may account for impaired ISG protein expression in Rictor KO cells. We performed reporter assays using a luciferase construct that included the WT ISG15 interferon-stimulated response

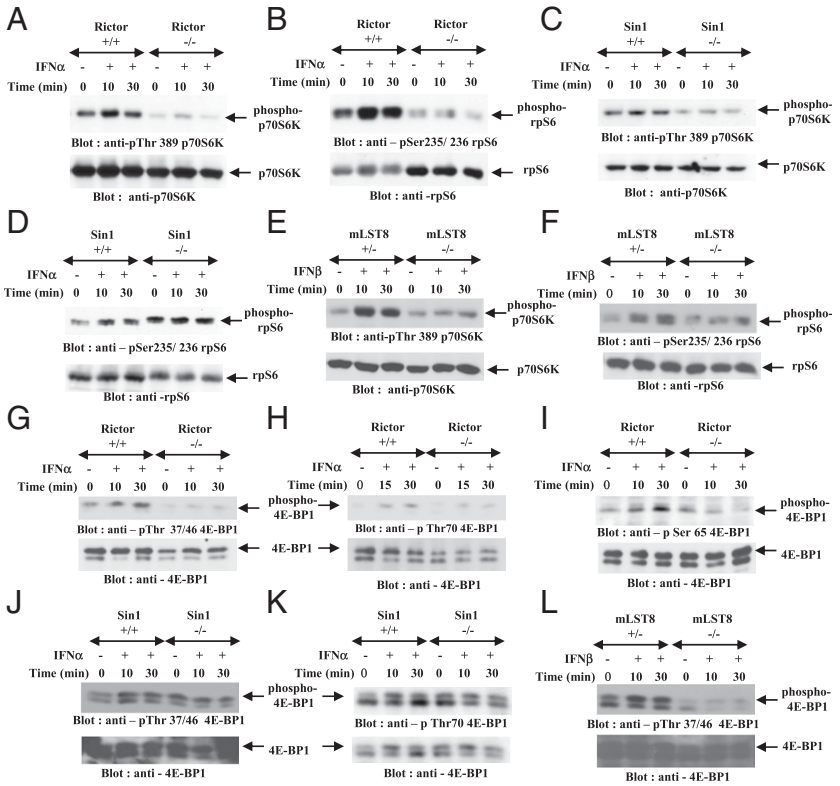

Fig. 2. Requirement of Rictor, Sin1, and mLST8 in type I IFN signaling. (*A* and *B*) Rictor$^{+/+}$ or Rictor$^{-/-}$ MEFs were treated with mouse IFN-α for the indicated times. Equal protein amounts were subjected to immunoblot analysis with anti–phospho-Thr-389 p70S6K (*A*) or anti–phospho Ser-235/236 rpS6 (*B*) antibodies. Respective blots were stripped and reprobed with anti-p70S6K (*A*, *Lower*) or anti-rpS6 (*B*, *Lower*) antibodies as indicated. (*C* and *D*) Sin1$^{+/+}$ or Sin1$^{-/-}$ MEFs were treated with mouse IFN-α for the indicated times. Equal protein aliquots were subjected to immunoblot analysis with anti–phospho-Thr-389 p70S6K (C) or anti–phospho-Ser-235/236 rpS6 (*D*) antibodies. Respective blots were stripped and reprobed with anti-p70S6K (*C*, *Lower*) or anti-rpS6 (*D*, *Lower*) antibodies as indicated. (*E* and *F*) mLST8$^{+/-}$ or mLST8$^{-/-}$ MEFs were treated with mouse IFN-β for the indicated times. Equal protein amounts were subjected to immunoblot analysis with anti–phospho-Thr-389 p70S6K (*E*) or anti–phospho-Ser-235/236 rpS6 (*F*) antibodies. Lysates from the same experiment were resolved on separate SDS/PAGE and immunoblotted with anti-p70S6K (*E*, *Lower*) or anti-rpS6 (*F*, *Lower*) antibody. (*G–I*) Rictor$^{+/+}$ or Rictor$^{-/-}$ MEFs were treated with mouse IFN-α for the indicated times. Equal protein amounts were subjected to immunoblot analysis with anti–phospho-Thr-37/46 4E-BP1 (*G*), anti–phospho-Thr70 4E-BP1 (*H*), or anti–phospho-Ser65 4E-BP1 (*I*) antibodies. *Lower*: Equal amounts of lysates from the same respective experiments were resolved separately by SDS/PAGE and probed with an anti–4E-BP1 antibody and are shown as indicated. (*J* and *K*) Sin1$^{+/+}$ or Sin1$^{-/-}$ MEFs were treated with mouse IFN-α for the indicated times. Equal protein amounts were subjected to immunoblot analysis with anti–phospho-Thr-37/46 4E-BP1 (*J*) or anti–phospho-Thr70 4E-BP1 (*K*) antibodies. The blots in the respective upper panels were stripped and probed with an anti–4E-BP1 antibody and shown in respective bottom panels as indicated. (*L*) mLST8$^{+/-}$ or mLST8$^{-/-}$ MEFs were treated with mouse IFN-β for the indicated times. Equal protein amounts were subjected to immunoblot analysis with anti–phospho-Thr-37/46 4E-BP1. The same blot was stripped and probed with an anti-4E-BP1 antibody and is shown as indicated (*Lower*).

element (ISRE) promoter element. We provide evidence for induction of luciferase activity in parental Rictor$^{+/+}$ and mLST8$^{+/-}$ MEFs in response to type I IFN treatment, but this induction was clearly decreased in Rictor$^{-/-}$ and mLST8$^{-/-}$ MEFs (Fig. 5 *A–C*). Taken together, these reporter assays suggested that mTORC2 complexes regulate downstream cellular pathways that are ultimately required for optimal transcription of ISGs. As our studies indicated impaired IFN-inducible engagement of mTOR effectors required for mRNA translation of ISGs in Rictor$^{-/-}$ cells, we sought to determine whether defects consistent with impaired ISG mRNA translation are noticeable in Rictor$^{-/-}$ MEFs. Rictor$^{+/+}$ and Rictor$^{-/-}$ MEFs were treated with IFN-α, polysomal mRNA was fractionated (Fig. 5*D*), and quantitative real-time RT-PCR was used to determine the amount of ISG15 mRNA in polysome fractions. ISG15 mRNA levels were decreased in Rictor$^{-/-}$ MEFs compared with Rictor$^{+/+}$ MEFs (Fig. 5*E*), indicating a reduction of ISG15 mRNA in polysomes in the absence of Rictor. There was also a reduction in induction of total ISG15 mRNA (Fig. 5*F*), consistent with the observed decreased inducible gene transcription via ISRE elements in reporter assays (Fig. 5 *A* and *B*). Thus, although the decrease in polysomal ISG15 mRNA is consistent with the impaired engagement of mTOR effectors whose function is required for initiation of mRNA translation (Fig. 2), it may reflect the decrease in total mRNA translation that apparently results from impaired transcription.

As our data demonstrated defective IFN-signaling events in Rictor KO cells, we compared induction of IFN-dependent antiviral responses in Rictor$^{-/-}$ and Rictor$^{+/+}$ cells. We found that Rictor$^{-/-}$ MEFs were highly sensitive to the cytopathic effects of encephalomyocarditis virus (EMCV) compared with parental cells; lower infective doses of virus were required to induce comparable cytopathic effects compared with Rictor$^{+/+}$ MEFs. Whereas the Rictor$^{+/+}$ MEFs responded to the protective effects of IFN-α treatment in a dose-dependent manner, identical IFN-α doses provided very weak protection in the Rictor$^{-/-}$ MEFs, despite being infected with lower doses of EMCV (Fig. 5*G*). Similar studies, to address the role of mLST8 in IFN-inducible antiviral response, revealed that mLST8$^{-/-}$ MEFs exhibited a weaker IFN-induced antiviral response as compared with their mLST8$^{+/-}$ counterparts (Fig. 5*H*). Taken together, these studies established that the mTORC2 complex plays a critical role in the generation of IFN-induced antiviral responses, a finding consistent with the observed defective expression of ISG proteins in cells with disrupted mTORC2 formation.

Discussion

It is well established that the AKT/mTOR pathway is engaged subsequent to the activation of IFN receptors, and accumulating evidence points to important roles for this cascade in the generation of the biological effects of IFNs (6, 17). Previous studies have shown that IFNs induce the formation of translation initiation complexes by activating mTOR (13). In studies that used cells with targeted disruption of the 4E-BP1 and TSC genes, it was shown that TSC1/2 and the translational repressor 4E-BP1 are negative upstream and downstream effectors, respectively, of IFN-activated mTOR, whereas expression of ISG15, CXCL10, and IFN-induced antiviral responses were enhanced in cells lacking 4E-BP1 or TSC2 (13). Other work has demonstrated that generation of IFN-dependent mTOR (mTORC1) signals and IFN-inducible cap-dependent mRNA translation are regulated upstream by AKT kinases, which are engaged by the type I and II IFN receptors (14). Remarkably, IFN-dependent antiviral responses are defective in the absence of AKT 1/2 (14), providing evidence that, beyond antiapoptotic responses, the AKT pathway promotes an IFN-antiviral state.

The initial demonstration that the mTOR pathway is activated by IFNs (11) was surprising and somewhat unexpected, as mTOR is a regulator of cap-dependent mRNA translation for oncogenic genes and mediates cell proliferation signals. Understanding the differences and mechanisms of specificity accounting for mTOR-

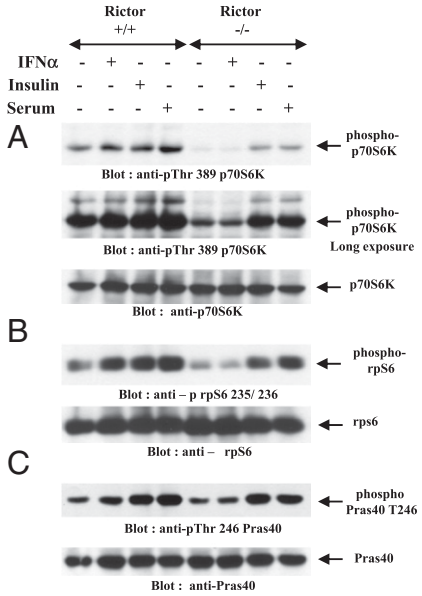

Fig. 3. Genetic disruption of Rictor impairs type I IFN-dependent, but not insulin- or serum-induced, phosphorylation of p70S6K or rpS6. (*A–C*) Rictor$^{+/+}$ or Rictor$^{-/-}$ MEFs were starved overnight in DMEM containing 0.5% FBS and treated with IFN-α, insulin, or serum (*Materials and Methods*). Equal protein amounts were subjected to SDS/PAGE and processed for immunoblot analysis with anti–phospho-Thr-389 p70S6K (*A*) or anti–phospho-Ser-235/236 rpS6 (*B*) antibody. Blots (*A*, *Top*; *B*, *Upper*) were stripped and probed with anti-p70S6K (*A*, *Bottom*) or anti-rpS6 (*B*, *Lower*) antibodies. A longer exposure of blot probed with anti–phospho-Thr-389 p70S6K is also shown (*A*, *Middle*). Lysates from the same experiment were also processed for immunoblot analysis with an anti–phospho-Thr-246 Pras40 antibody (*C*). The blot in *C*, *Upper* was stripped and probed with anti-Pras40 antibody, shown as indicated (*C*, *Lower*).

mediated responses to IFNs, which are cytokines with growth-inhibitory properties, vs. growth factors and oncogenic signals, has been challenging. To date, no differences have been identified on the patterns of phosphorylation/mechanisms of engagement of downstream mTOR effectors. However, the identification of specific ISGs as target products of the mTOR cascade (13–17) has suggested that coordination of activation of JAK–STAT pathways that control IFN-dependent gene transcription and simultaneous engagement of AKT/mTOR pathways provide an integrated signaling network for generation of IFN-inducible proteins that mediate antiviral and antiproliferative responses.

mTOR exists in two distinct complexes, mTORC1 and mTORC2 (24, 29, 36). Both complexes share mTOR as their catalytic subunit and comprise common and distinct elements. mTORC1 includes mTOR, Raptor, and mLST8 in its structure, whereas mTORC2 includes mTOR, Rictor, mLST8, and Sin1 (24, 36). mTORC1 complexes are targeted by classic mTOR inhibitors such as rapamycin or RAD001, whereas new catalytic inhibitors of mTOR target and inhibit mTORC1 and mTORC2 complexes (37). Because mTORC2 regulates phosphorylation and activation of AKT, which mediates antiapoptotic responses, these complexes are considered good targets for new anticancer agents aimed to block antiapoptotic responses in malignant cells. Indeed, dual mTORC1/mTORC2 inhibitors exhibit more potent antileukemic effects in vitro and in vivo compared with specific mTORC1 targeting agents, underscoring the importance of mTORC2 complexes in proliferation and survival of malignant cells (38–40). Although the sequence of events for activation of mTORC1 complexes has been well established (25), relatively little is known about the mechanisms of mTORC2 activation. Recent evidence has suggested that mTORC2 can be activated in a PI3 kinase-dependent manner under certain circumstances (41). Notably, in previous work, we demonstrated that the PI3 kinase pathway is also engaged by type I IFN receptors (42) and ultimately regulates cap-dependent mRNA translation by controlling the mTOR pathway (16). There are also recent reports providing evidence that association with ribosomes is essential for activation of mTORC2 complexes (43, 44).

As the function of mTORC2 in the IFN system has been unknown, we sought to identify the roles of mTORC2 complexes in type I IFN signaling and biological responses. By using MEFs with genetic disruption of three different components of the mTORC2

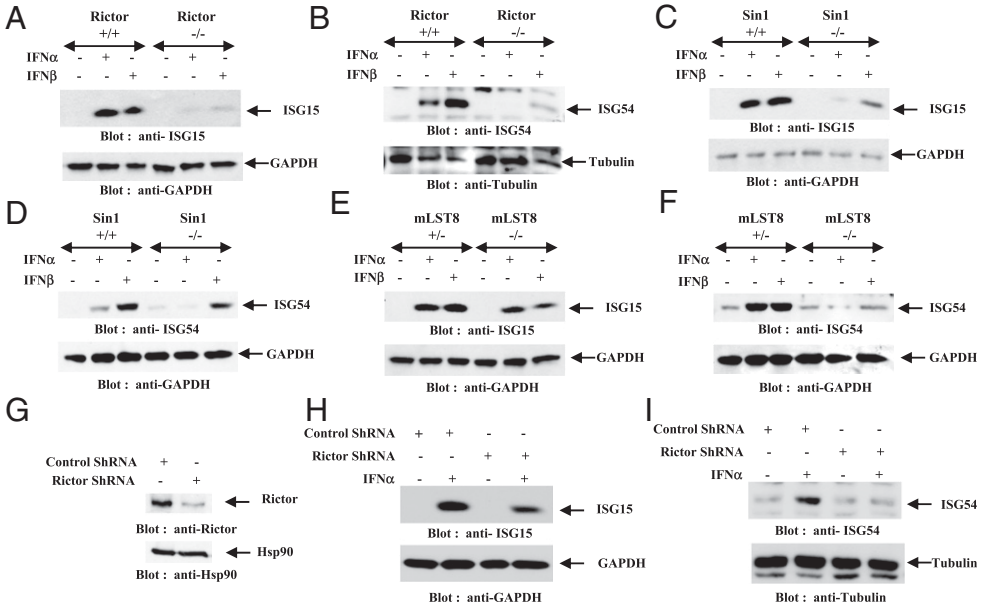

Fig. 4. Type I IFN-induced ISG15 and ISG54 expression is mTORC2-dependent. (*A* and *B*) Rictor$^{+/+}$ and Rictor$^{-/-}$ MEFs were treated with mouse IFN-α or IFN-β for 24 h as indicated. Equal protein amounts were resolved by SDS/PAGE and probed with anti-ISG15 (*A*) or anti-ISG54 (*B*) antibodies. Respective blots were reprobed with anti-GAPDH (*A*, *Lower*) or antitubulin (*B*, *Lower*) antibodies, as indicated, to control for protein loading. (*C* and *D*) Sin1$^{+/+}$ and Sin1$^{-/-}$ MEFs were treated with mouse IFN-α or IFN-β for 24 h as indicated. Equal protein amounts were resolved by SDS/PAGE and probed with anti-ISG15 (*C*) or anti-ISG54 (*D*) antibodies. Respective blots were probed with anti-GAPDH antibody (*Lower*) to control for protein loading. (*E* and *F*) mLST8$^{+/-}$ and mLST8$^{-/-}$ MEFs were treated with mouse IFN-α or IFN-β for 24 h as indicated. Equal protein amounts were resolved by SDS/PAGE and probed with anti-ISG15 (*E*) or anti-ISG54 (*F*) antibodies. Respective blots were probed with anti-GAPDH antibodies (*Lower*) to control for protein loading. (*G–I*) Cell lysates from U937 cells stably infected with lentiviral control shRNA or Rictor shRNA were immunoblotted with an anti-Rictor antibody (*G*, *Upper*). The same blot was probed with an anti-Hsp90 antibody to control for protein loading (G, *Lower*). U937 cells stably infected with lentiviral control shRNA or Rictor ShRNA were treated with human IFN-α as indicated. Equal protein aliquots were processed for immunoblotting with anti-ISG15 (*H*) or anti-ISG54 (*I*) antibodies. The same blots were reprobed with anti-GAPDH (*H*, *Lower*) or antitubulin (*I*, *Lower*) antibodies to control for protein loading.

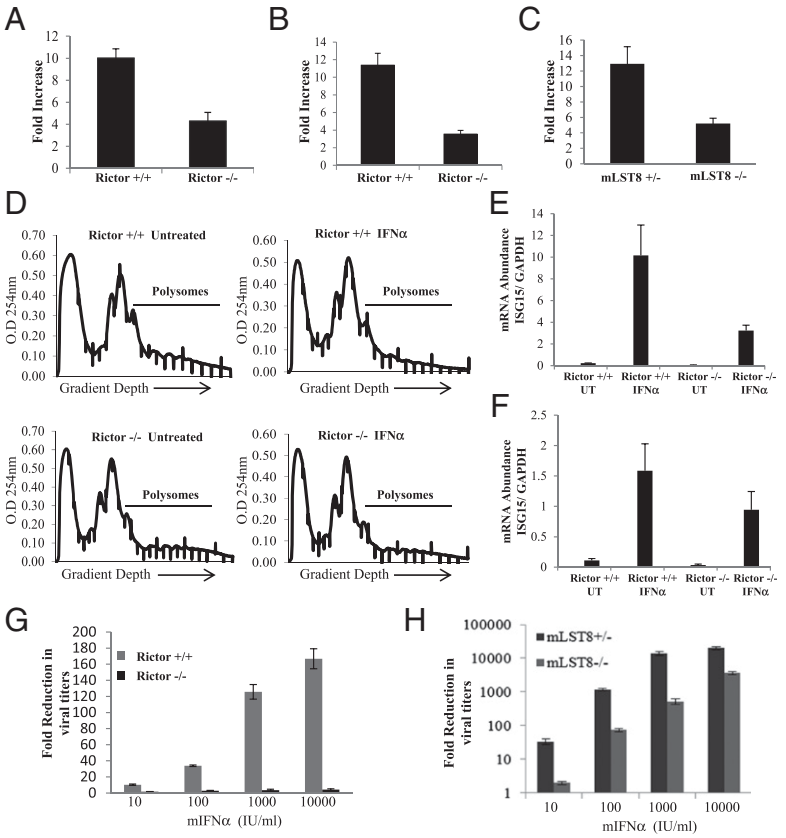

Fig. 5. Essential role of mTORC2 complexes in the generation of IFN responses. (*A* and *B*) Rictor$^{+/+}$ and Rictor$^{-/-}$ MEFs were transfected with ISRE luciferase and β-gal expression plasmids. Cells were left untreated or treated with IFN-α (*A*) or IFN-β (*B*) for 6 h, and luciferase reporter assays were performed. Data are expressed as fold increase of luciferase activity in IFN-treated samples vs. untreated samples, and represent means ± SE of three experiments with IFN-α (*A*) and means ± SE of four experiments with IFN-β (*B*). (*C*) mLST8$^{+/-}$ and mLST8$^{-/-}$ MEFs were transfected with ISRE luciferase and β-gal expression plasmids. Cells were left untreated or treated with IFN-β, and luciferase reporter assays were performed. Data are expressed as fold increase of luciferase activity in IFN-β–treated samples vs. untreated samples and represent means ± SE of four experiments. (*D*) Rictor$^{+/+}$ and Rictor$^{-/-}$ MEFs were left untreated or treated with 2,500 IU/mL of mouse IFN-α in DMEM containing 0.5% FBS. Cell lysates were layered on 10% to 50% sucrose gradient and subjected to density gradient centrifugation, and fractions were collected by continuous monitoring of OD at 254 nm. OD_{254} is shown as a function of gradient depth, and the polysomal fractions are indicated. (*E*) Polysomal fractions were pooled and RNA was isolated. Subsequently, quantitative real-time RT-PCR was carried out to determine ISG15 mRNA expression in polysomal fractions, using GAPDH for normalization. Data are expressed as mRNA abundance of ISG15/GAPDH in each sample and represent means ± SE of seven independent experiments. (*F*) Rictor$^{+/+}$ and Rictor$^{-/-}$ MEFs were left untreated or treated with 2,500 IU/mL of mouse IFN-α for 24 h, and total RNA was isolated. Expression of ISG15 mRNA was evaluated by quantitative real-time PCR, and GAPDH was used for normalization. Data are expressed as mRNA abundance of ISG15/GAPDH in each sample and represent means ± SE of seven independent experiments. (*G*) Rictor$^{+/+}$ and Rictor$^{-/-}$ MEFs were treated with the indicated doses of IFN before infection with EMCV. Rictor$^{+/+}$ MEFs were infected with an MOI of 1 and Rictor$^{-/-}$ MEFs with an MOI of 0.01, and incubated for 17 h. Culture medium containing the virus was then collected and viral titers were determined by standard plaque assay in HeLa cells. Data are expressed as fold reduction in viral titers relative to untreated cells and represent means ± SE of two independent experiments. (*H*) mLST8$^{+/-}$ and mLST8$^{-/-}$ MEFs were treated with the indicated doses of IFN before infection with EMCV at an MOI of 0.01 and were incubated for 17 h. Culture medium containing the virus was then collected, and viral titers were determined by standard plaque assay in HeLa cells. The experiment shown is representative of five independent experiments, and data represent means ± SE.

complex, namely Rictor, Sin1, and mLST8, we assessed their roles in IFN-signaling events. IFN-induced phosphorylation of AKT on Ser-473 was defective in Rictor$^{-/-}$, Sin1$^{-/-}$, and mLST8$^{-/-}$ cells, establishing that mTORC2 is required for AKT engagement in response to type I IFNs. Importantly, we noticed decreased or defective phosphorylation of downstream mTORC1 effectors, such as S6K, rpS6, and 4E-BP1, likely reflecting the requirement for AKT activity (14) in the generation of these type I IFN-signaling events. In other studies, we found that inducible expression of ISG15 and ISG54 proteins is defective in the absence of intact mTORC2 complexes. Further studies established the presence of a transcriptional defect for ISRE elements in Rictor$^{-/-}$ cells, suggesting a mechanism for the defective ISG expression. Although the observed decreased ISG mRNA expression in polysomal fractions is difficult to interpret in the context of simultaneously defective gene transcription, it is possible that ISG mRNA translation is also affected in cells with disrupted mTORC2 complexes, as suggested by the decreased IFN-induced phosphorylation of S6K and 4E-BP1 in these cells. In the absence of specifics relating to the precise mechanism, our data established that defective ISG protein expression in Rictor-KO cells results in defective antiviral responses.

Beyond establishing an important role for mTORC2 complexes in the generation of IFN responses, the results of this study suggest a unique function of mTORC2 in the IFN system, distinct from its function in growth factor signaling and proliferative responses. Previous reports have shown that genetic KO of Rictor does not affect growth factor-dependent activation of S6K and rpS6 (27, 30). Beyond Rictor, Sin1 is required for AKT stability and folding, and, as a part of mTORC2 complexes (45), the function of Sin1 is required for phosphorylation of AKT on Ser-473 (31). On the contrary, insulin- and serum-induced phosphorylation of S6K and 4E-BP1 phosphorylation on Thr-37/46 was not blocked in Sin1-KO cells (31). In studies using MEFs from two different Rictor KOs (27, 30), we found that IFN-inducible phosphorylation of S6K on Thr-389 and rpS6 on Ser-235/236 was completely defective or very weak in Rictor$^{-/-}$ MEFs compared with phosphorylation in response to insulin or serum as analyzed in parallel. The reasons for these differences are not clear, but these data establish a relative selectivity and specificity in the IFN system that may account for differential responses invoked by IFNs vs. growth factors. Although the precise mechanisms of differential mTORC2-controlled signaling in response to IFNs vs. oncogenic signals remain to be defined, these results raise the potential of future therapeutic exploitation of these differences to selectively target malignant cells.

Materials and Methods

Cell Lines and Reagents. Immortalized MEFs were grown in DMEM supplemented with 10% FBS and gentamycin. Immortalized Rictor$^{+/+}$ (rictor $^{Ex3cond/w}$) and Rictor$^{-/-}$ (rictor $^{Ex3del/Ex3del}$) MEFs were provided by Mark Magnuson (Vanderbilt University, Nashville, TN) (30). Some of the studies were confirmed using immortalized Rictor$^{+/+}$ (control) and Rictor$^{-/-}$ (rictor-null) MEFs provided by David Sabatini (Whitehead Institute, Cambridge, MA) (27). Immortalized Sin1$^{+/+}$ and Sin1$^{-/-}$ MEFs (31) were also used. Immortalized mLST8$^{+/-}$ and mLST8$^{-/-}$ MEFs were provided by David Sabatini (Whitehead Institute, Cambridge, MA) (27).

Cell Lysis and Immunoblotting. The indicated immortalized MEFs were starved overnight in DMEM containing 0.5% FBS and then treated with the indicated IFNs in DMEM containing 0.5% FBS. For parallel studies involving comparisons of treatments with IFN-α, insulin, or serum, cells were starved overnight in DMEM containing 0.5% FBS, followed by treatment with 5×10^3 IU/mL of IFN, 200 nM insulin, or 10% FBS for 15 min. Following treatments, cells were

washed with PBS solution and lysed in phosphate lysis buffer containing protease and phosphatase inhibitors as described previously (13, 16). The lysates were resolved by SDS/PAGE and immunoblotted, and enhanced chemiluminescence was carried out as described previously (13, 16).

Luciferase Reporter Assays. Immortalized Rictor$^{+/+}$ and Rictor$^{-/-}$ or immortalized mLST8$^{+/-}$ and mLST8$^{-/-}$ MEFs were transfected with a β-gal expression vector and an ISRE-luciferase plasmid by using the SuperFect transfection reagent (Qiagen). Forty-eight hours after transfection, triplicate cultures were left untreated or treated with IFN-α or IFN-β as indicated. Luciferase activity was measured as previously described (46). Luciferase activities were normalized for β-gal activity for each sample.

Polysomal Fractionation, RNA Isolation, and RT-PCR. Immortalized Rictor$^{+/+}$ and Rictor$^{-/-}$ MEFs were left untreated or treated with 2500 IU/mL of IFN-α for 24 h in DMEM supplemented with 0.5% FBS. The samples for total RNA isolation were processed by using the RNAeasy kit from Qiagen. For polysomal isolation, samples were layered on sucrose gradients to isolate polysomal fractions as described previously (14–16). Polysomal RNA was isolated using the RNA AllPrep kit from Qiagen. Total or polysomal RNA was reverse-transcribed by using oligodT primers and the Omniscript RT PCR kit from Qiagen. Real-time PCR was carried out by using custom-made ISG15 and GAPDH primers and SYBR Green mix (Applied Biosystems). In each 96-well plate, a dilution series of the cDNA standard for ISG15 and GAPDH was run along with the unknown samples. After SYBR Green PCR amplification, data acquisition and subsequent data analyses were carried out by using 7500 sequence detection software (Applied Biosystems). The amount of ISG15 and GAPDH cDNA was estimated in each sample by using a SYBR green standard curve, and results were plotted as a ratio of ISG15/GAPDH for each sample.

Antiviral Assays. For plaque assays, immortalized Rictor$^{+/+}$ and Rictor$^{-/-}$ MEFs or mLST8$^{+/-}$ and mLST8$^{-/-}$ MEFs were plated at 1×10^5 cells per well of a 24-well plate in DMEM containing 2% FBS. Twenty-four hours later, the cells were left untreated or treated with mouse IFN-α at 10, 100, 1,000, and 10,000 IU/mL for 8 h, before infection with EMCV. Immortalized Rictor$^{+/+}$ MEFs were infected with at a multiplicity of infection (MOI) of 1 and Rictor$^{-/-}$ MEFs with an MOI of 0.01. mLST8$^{+/-}$ and mLST8$^{-/-}$ were infected with an MOI of 0.01. The virus was allowed to absorb for 60 min, after which the cells were washed three times with DMEM supplemented with 2% FBS and incubated for an additional 17 h. Medium containing virus was then collected and viral titers were determined by standard plaque assay in HeLa cells. A total of 10^6 HeLa cells were seeded 24 h before infection with 500 μL of medium containing log-fold dilutions of the virus in serum-free RPMI 1640 medium. After 60 min, 2 mL of medium with agar was added and allowed to solidify, and the plates were incubated at 37 °C in 5% CO_2 for 24 h. Cells were fixed with Carnoy fixative and stained with crystal violet, and plaques were counted. Data are expressed as fold reduction in viral titers relative to untreated cells.

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