Protein kinase IKK β -catalyzed phosphorylation of IRF5 at Ser462 induces its dimerization and nuclear translocation in myeloid cells

Marta Lopez-Pelaez^a, Douglas J. Lamont^b, Mark Peggie^a, Natalia Shpiro^a, Nathanael S. Gray^c, and Philip Cohen^{a,1}

^aMedical Research Council Protein Phosphorylation and Ubiquitylation Unit, and ^bCollege of Life Sciences, University of Dundee, Dundee DD1 5EH, Scotland, United Kingdom; and ^cDana-Farber Cancer Institute, Harvard Medical School, Boston, MA 02215

Contributed by Philip Cohen, September 24, 2014 (sent for review June 30, 2014; reviewed by Vishva M. Dixit, Michael Karin, and Giulio Superti-Furga)

The siRNA knockdown of IFN Regulatory Factor 5 (IRF5) in the human plasmacytoid dendritic cell line Gen2.2 prevented IFN β production induced by compound CL097, a ligand for Toll-like receptor 7 (TLR7). CL097 also stimulated the phosphorylation of IRF5 at Ser462 and stimulated the nuclear translocation of wild-type IRF5, but not the IRF5[Ser462Ala] mutant. The CL097-stimulated phosphorylation of IRF5 at Ser462 and its nuclear translocation was prevented by the pharmacological inhibition of protein kinase IKK β or the siRNA knockdown of IKK β or its "upstream" activator, the protein kinase TAK1. Similar results were obtained in a murine macrophage cell line stimulated with the TLR7 agonist compound R848 or the nucleotide oligomerization domain 1 (NOD1) agonist KF-1B. IKK β phosphorylated IRF5 at Ser462 in vitro and induced the dimerization of wild-type IRF5 but not the IRF5[S462A] mutant. These findings demonstrate that IKK β activates two "master" transcription factors of the innate immune system, IRF5 and NF- κ B.

IKK β | interferon β | IRF5 | plasmacytoid dendritic cell | TLR7

The transcription factor IFN Regulatory Factor 5 (IRF5) has a critical role in the production of proinflammatory cytokines. The secretion of interleukin 12 (IL-12), IL-6, and TNF α by ligands that activate Toll-like receptor 3 (TLR3), TLR4, TLR5, and TLR9, is greatly impaired in macrophages, conventional dendritic cells (cDCs), and plasmacytoid dendritic cells (pDCs) of mice that do not express IRF5 (1). However, the role of IRF5 in type 1 IFN production in pDCs has been less clear. The TLR9-stimulated secretion of IFN α was initially reported to be similar in pDCs from IRF5-deficient and wild-type mice (1), but a later study found that the TLR7- or TLR9-stimulated production of IFN α was reduced in pDCs from IRF5-deficient mice (2). Subsequently, some IRF5-deficient mouse lines were shown to carry a second mutation in the gene encoding the guanine nucleotide exchange factor (dedicator of cytokinesis 2 (Dock2)), and it was reported that TLR9-stimulated IFN α secretion was largely intact in pDCs from mice where this secondary mutation had been eliminated (3). A further study using pDCs from IRF5-deficient mice, carrying or not carrying the DOCK2 mutation, confirmed that IRF5 had little effect on TLR9-stimulated IFN α secretion, but indicated an important role for IRF5 in the secretion of IFN β (4). IRF5 was also required for the TLR9-stimulated production of IFN β in the human pDC line CAL-1 (5) and for the production of IFN β induced by streptococcal RNA in cDCs (6) and by the fungal pathogen *Candida albicans* in a pathway dependent on the Dectin 1 and Dectin 2 receptors (7). IRF5 was also found to be needed for the production of IFN β by a small subset of viruses (8, 9). Thus, IRF5 does appear to be a key regulator of IFN β production by several pathogens.

IRF5 is present in a latent form in the cell cytosol but accumulates in the nucleus to stimulate gene transcription following viral infection (10, 11) or stimulation with the TLR7/TLR8 agonist R848 (9). The nuclear export signal (NES) (12) of IRF5 therefore appears to be dominant over the two nuclear localization signals (8) in uninfected/unstimulated cells. R848 also stimulated the

transcription of an IRF5 reporter gene in HEK293 cells over-expressing TLR7 or TLR8, and this was accompanied by the translocation of an IRF5-GFP fusion protein from the cytosol to the nucleus (9). Taken together, these findings indicated that IRF5-dependent gene transcription requires the translocation of the transcription factor to the nucleus.

The production of IFN β triggered by ligands that activate TLR3 and TLR4, or by viruses that form double-stranded (ds) RNA during their replication, does not depend on IRF5, but instead requires the phosphorylation of IRF3 catalyzed by the I κ B kinase (IKK)-related kinase TANK-binding kinase 1 (TBK1) (13–15). TBK1 was reported to phosphorylate a GST-IRF5 fusion protein in vitro, whereas IKK β did not (9), and the TLR7-stimulated activation of a Gal4-IRF5 reporter gene was inhibited by the over-expression of a catalytically inactive mutant of TBK1 or the related IKK ϵ . Based on these experiments, it was suggested that TBK1/IKK ϵ might activate IRF5 as well as IRF3. Two serines in IRF5, Ser158 and Ser309, were subsequently identified as amino acid residues that became phosphorylated when DNA vectors encoding IRF5 and TBK1 were coexpressed in cells (16).

Here we demonstrate that the TLR7 agonist CL097 induces a striking increase in the phosphorylation of the endogenous IRF5 at Ser462 in the human pDC cell line Gen2.2 and establish that the phosphorylation of this site is required for the dimerization and nuclear translocation of IRF5. We also show that phosphorylation of Ser462 is needed for the nuclear translocation of IRF5 in the macrophage cell line RAW264.7 by the TLR7 agonist R848 or

Significance

NF- κ B and IFN Regulatory Factor 5 (IRF5) are required for the transcription of many proinflammatory cytokines in myeloid cells. The protein kinase IKK β is the major activator of NF- κ B but how IRF5 is activated has been unclear. This paper demonstrates that IKK β also activates IRF5 by catalyzing the phosphorylation of Ser462. The phosphorylation of this serine induces the dimerization of IRF5 and its translocation to the nucleus. The activation of the master transcription factors of the innate immune system by the same protein kinase provides a mechanism for the coordinated control of IRF5 and NF- κ B in response to inflammatory stimuli.

Author contributions: M.L.-P. and P.C. designed research; M.L.-P. performed research; D.J.L., M.P., N.S., and N.S.G. contributed new reagents/analytic tools; M.L.-P. and P.C. analyzed data; and M.L.-P. and P.C. wrote the paper.

Reviewers: V.M.D., Genentech; M.K., University of California, San Diego School of Medicine; and G.S.-F., CeMM Research Center for Molecular Medicine of the Austrian Academy of Sciences.

The authors declare no conflict of interest.

Freely available online through the PNAS open access option.

See Commentary on page 17348.

¹To whom correspondence should be addressed. Email: p.cohen@dundee.ac.uk.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1418399111/-DCSupplemental.

the NOD1 agonist KF-1B. Unexpectedly, we demonstrate that IKK β is the protein kinase that phosphorylates IRF5 at Ser462 in myeloid cells.

Results

IRF5 Is Required for IFN β Production in Gen2.2 Cells. It is widely accepted that the high level of expression of the transcription factor IRF7 in pDCs underlies the ability of these cells to produce large amounts of type 1 IFNs in response to ligands that activate TLR7 or TLR9 (17). In view of emerging evidence that IRF5 may be important for the production of IFN β (see Introduction) we decided to reinvestigate the relative importance of IRF5 and IRF7 in stimulating transcription of the *IFN β* and *IFN α 1* genes in Gen2.2 cells, which is triggered by stimulation with the TLR7 ligand CL097 (18). We found that the siRNA “knockdown” of IRF5 (Fig. S14) prevented the CL097-stimulated production of IFN β mRNA (Fig. 1A) or IFN β secretion (Fig. 1B). In contrast, the CL097-stimulated production of IFN α 1 mRNA in human Gen 2.2 cells was consistently enhanced by the siRNA knockdown of IRF5 (Fig. 1C). The secretion of IFN α could not be determined as it was below the level that can be detected by ELISA. These findings contrast with earlier reports that the TLR9-stimulated secretion of IFN α was little affected in pDCs from IRF5-deficient mice (1, 3, 4). Control experiments showed that the siRNA knockdown of IRF5 in Gen2.2 cells greatly decreased the production of IL-12 mRNA (Fig. S1B and C), in line with earlier studies performed in other immune cells (see Introduction). However, the TLR7-stimulated production of IL-6 mRNA in Gen2.2 cells was unaffected by the knockdown of IRF5 (Fig. 1D). This result also differs from an earlier report in which the TLR9-stimulated production of IL-6 mRNA was suppressed in spleen-derived pDCs from IRF5 knockout mice (1).

In contrast to IRF5, the siRNA knockdown of IRF7 (Fig. S14) prevented the induction of IFN α 1 mRNA (Fig. 1C) and reduced the production of IFN β mRNA significantly (Fig. 1A). Consistent with the latter finding, the secretion of IFN β was decreased

by 50% at each time point examined (Fig. 1B). The knockdown of IRF7 did not affect the production of IL-6 (Fig. 1D), IL-12p35 (Fig. S1B), or IL-12p40 significantly (Fig. S1C). Taken together, our results support the emerging consensus (see Introduction) that IRF5 has a critical role in the production of IFN β , whereas IRF7 is critical for the production of IFN α . In overexpression experiments IRF5 and IRF7 form heterodimers and the IRF5/IRF7 heterodimer is reported to be less active than the IRF7 homodimer in stimulating IFN α gene transcription (19). This could explain why the knockdown of IRF5 enhances the CL097-stimulated production of IFN α 1 mRNA. Heterodimer formation might also explain why the IRF5-dependent production of IFN β is enhanced by IRF7, if the IRF5/IRF7 heterodimer is more efficient than the IRF5 homodimer in stimulating IFN β gene transcription. The knockdown of IRF3 (Fig. S14) did not affect the CL097-stimulated production of IFN α , IFN β , or any other cytokine measured significantly (Fig. 1 and Fig. S1).

The CL097-Stimulated Phosphorylation of IRF5 at Ser462 is Catalyzed by IKK β . The results presented in Fig. 1 raised the question of how IRF5 might be activated to stimulate IFN β gene transcription. We therefore used SILAC in conjunction with mass spectrometry as an unbiased approach to identify proteins that became phosphorylated when Gen2.2 cells were stimulated with CL097 (Fig. S24). A tryptic peptide with a molecular mass corresponding to amino acid residues 459–467 plus one phosphate group (the numbering corresponds to the longest alternatively spliced variant of IRF5) was detected in every experiment, and analysis of the fragment ions generated identified Ser462 as the site of phosphorylation, the only Ser/Thr residue in this peptide. The phosphorylation of Ser462 increased 27-fold $\pm$ 7.5-fold ($\pm$ SEM for 10 independent experiments) after stimulation for 30 min with CL097 (Fig. 2A). No other phosphorylation site in the endogenous IRF5 was detected in these experiments either before or after stimulation with CL097.

We have reported that the CL097-stimulated production of IFN β mRNA and IFN β secretion in Gen2.2 cells is prevented by the siRNA knockdown or pharmacological inhibition of IKK β (18). The molecular mechanism(s) was not identified in these studies, but was largely independent of the activation of the transcription factor NF- κ B. We therefore performed additional SILAC mass spectrometry experiments to investigate whether the phosphorylation of IRF5 at Ser462 was dependent on IKK β activity. We found that the CL097-stimulated phosphorylation of IRF5 was prevented by compound BI605906 (Fig. 2A and Fig. S2B and D), the most specific inhibitor of IKK β so far identified, which does not inhibit IKK α or the IKK-related kinases, termed TBK1 and IKK ϵ (20). Consistent with this observation, the phosphorylation of the endogenous IRF5 was also suppressed by compound NG-25 (Fig. 2A and Fig. S2C and D), a potent and relatively specific inhibitor of TAK1, the protein kinase that activates IKK β .

The suppression of IRF5 phosphorylation at Ser462 by inhibitors of IKK β or TAK1 did not establish that IKK β catalyzed the phosphorylation of Ser462 directly, because IKK β might have exerted its effect indirectly by phosphorylating and activating another protein kinase, which then catalyzed the phosphorylation of Ser462. Indeed IKK β was reported to be unable to phosphorylate IRF5 in vitro (9). In contrast, we found that purified IKK β and, to a lesser extent, IKK α did phosphorylate IRF5 in vitro (Fig. 2B). Moreover, IKK β and IKK α phosphorylated IRF5 at Ser462 (Fig. 2C), as judged by immunoblotting with an antibody that recognizes IRF5 phosphorylated at Ser462, but not the IRF5[S462A] mutant (Fig. 2D, Upper). Purified TBK1 phosphorylated purified IRF5 more robustly than IKK β or IKK α in vitro (Fig. 2B), but did not phosphorylate IRF5 at Ser462 (Fig. 2C). The IKK β -catalyzed phosphorylation of IRF5 was suppressed by BI605906 and the TBK1-catalyzed phosphorylation of IRF5 by compound

Fig. 1. IRF5 is required for CL097-stimulated IFN β production but not for IFN α 1 or IL-6 production in Gen2.2 cells. (A–D) Gen2.2 cells were transfected with siRNA against IRF3 (hatched bars), IRF5 (white bars), IRF7 (gray bars), or a control siRNA (black bars). After 72 h, the cells were stimulated for the times indicated with 1.0 μ g/mL CL097. The mRNA encoding IFN- β (A), IFN- α 1 (C), and IL-6 (D) was then measured in duplicate by qRT-PCR. Graphs show the fold increase in mRNA production relative to that measured in cells treated with control siRNA and not stimulated with CL097 and are presented as the mean $\pm$ SD for one representative experiment. The mRNA levels were normalized to 18S rRNA. (B) As in A, except that IFN β secreted into the culture medium was measured by ELISA. All experiments (A–D) were repeated three times with similar results.

Fig. 2. IRF5 is phosphorylated by IKK β at Ser462 in Gen2.2 cells and in vitro. (A) SILAC-labeled Gen2.2 cells were incubated without (–) or with (+) BI605906 or NG-25 stimulated for 30 min without (–) or with (+) 1.0 μ g/mL CL097 and the fold increase in the abundance of the tryptic phosphopeptide LQISpNDLK (where Sp is phosphoserine) corresponding to amino acid residues 459–467 of IRF5 was quantified as described in *Materials and Methods*, relative to the level in unstimulated cells (mean $\pm$ SEM). The number of independent experiments performed is indicated in parentheses. (B and C) Flag-IRF5 was phosphorylated with His6-IRK β , GST-IRK α , or GST-TBK1 in the presence or absence of BI605906 or MRT67307 using [γ - 32 P]ATP (B) or unlabeled ATP (C) and the incorporation of 32 P-radioactivity into proteins or phosphorylation of Ser462 of IRF5 examined by autoradiography (B, Upper) or immunoblotting (C). The gel in B was also stained with Coomassie blue. (D, Lower) HEK293T cells were transfected with DNA encoding wild-type (WT) HA-IRK β or the catalytically inactive HA-IRK β [D166A] mutant and either Flag-IRF5[WT] or the Flag-IRF5[S462A] mutant. The cell extracts were subjected to SDS/PAGE (Upper) or native gel electrophoresis (Lower two panels) and immunoblotted with the antibodies indicated. Similar results were obtained in three independent experiments.

MRT67307 (Fig. 2 B and C), a potent inhibitor of TBK1 and IKK ϵ that does not inhibit IKK β or IKK α (20). These control experiments demonstrated that the phosphorylation of IRF5 was catalyzed by IKK β and TBK1 and not by another kinase(s) that might have been present in the preparations as a contaminant.

The cotransfection of FLAG-IRF5 with HA-IRK β , but not the catalytically inactive mutant IKK β [D166A], induced the phosphorylation of IRF5 at Ser462 (Fig. 2D, Top) and the dimerization of IRF5 (Fig. 2D, Bottom). Importantly, only the dimeric IRF5 was phosphorylated at Ser462 (Fig. 2D, Middle), strongly suggesting that dimerization was triggered by the phosphorylation of Ser462. This was confirmed by the finding that the IRF5[S462A] mutant did not dimerize when cotransfected with IKK β (Fig. 2D, Bottom). These experiments also established the specificity of the antibody for the Ser462 phosphorylation site on IRF5 (Fig. 2D, Top).

The Phosphorylation of IRF5 at Ser462 Is Required for Nuclear Translocation. To study the role of Ser462 phosphorylation, we initially expressed DNA encoding an IRF5–GFP fusion protein

in Gen 2.2 cells. The IRF5–GFP was excluded from the nucleus but underwent partial translocation to the nucleus within 30 min of stimulation with CL097. In contrast, CL097 did not stimulate the nuclear translocation of the IRF5[Ser462Ala] mutant, suggesting a critical role for Ser462 phosphorylation in the nuclear accumulation of IRF5 (Fig. 3A and Fig. S3A).

The Nuclear Translocation of IRF5 Is Prevented by IKK β or TAK1 Inhibitors. Because IKK β phosphorylated IRF5 in Gen 2.2 cells (Fig. 2A) and in vitro (Fig. 2B and C), and the CL097-stimulated phosphorylation of Ser462 was required for the nuclear translocation of IRF5 in Gen2.2 cells (Fig. 3A and Fig. S3A), we investigated whether nuclear translocation of IRF5 was prevented by the inhibition of IKK β or its upstream activator TAK1. We found that the CL097-stimulated nuclear translocation of IRF5 was prevented by two structurally unrelated inhibitors of IKK β , BI605906 and compound PS1145 or by the TAK1 inhibitor NG-25 (Fig. 4A and Fig. S3B). In contrast, the TBK1/IKK ϵ inhibitor MRT67307 did not block the nuclear translocation of IRF5 (Fig. 4A and Fig. S3B) at a concentration that inhibited the

Fig. 3. The mutation of Ser462 to Ala prevents the nuclear translocation of IRF5 induced by TLR7 agonists. (A) Gen2.2 cells were transfected with IRF5–GFP or IRF5[S462A]–GFP. After 48 h, the cells were stimulated for 30 min with 1.0 μ g/mL CL097 or left unstimulated. The cells were fixed, centrifuged onto precoated slides, permeabilized, and stained with anti-GFP or DAPI to reveal nuclei. (B) Same as A, except that RAW264.7 cells were stimulated with R848 (1.0 μ g/mL).

Fig. 4. The nuclear translocation of IRF5-GFP is prevented by inhibitors of IKK β (BI605906 and PS1145) or TAK1 (NG-25). (A) As in Fig. 3 except that Gen2.2 cells transfected with IRF5-GFP were incubated for 1 h without or with BI605906 (5.0 μ M), PS1145 (15 μ M), NG-25 (2.0 μ M), or MRT67307 (2.0 μ M), then stimulated without or with CL097 (1.0 μ g/mL). (B) Same as A, except that RAW264.7 cells were stimulated with R848.

lipopolysaccharide/TLR4-stimulated phosphorylation of IRF3 at Ser396 in macrophages (Fig. S4), which is dependent on TBK1/IKK ϵ catalytic activity (20).

Overexpression studies can sometimes cause the specificity of signaling to break down and we therefore also studied the nuclear accumulation of the endogenous IRF5. These experiments showed that a small proportion of the endogenous IRF5 underwent nuclear translocation in response to CL097, which was decreased by the IKK β inhibitors BI605906 and PS1145, and even more strongly by TAK1 inhibitor NG-25, whereas the TBK1/IKK ϵ inhibitor MRT67307 was without effect (Fig. 5A). The CL097-stimulated nuclear translocation of IRF5 was also suppressed by siRNA knockdown of IKK β (Fig. 5B). Interestingly, the knockdown of IKK α did not affect the CL097-stimulated nuclear accumulation of IRF5 significantly, but in combination with the IKK β inhibitor BI605906 completely prevented the nuclear accumulation of IRF5 (Fig. 5C). Similar observations were made when the nuclear accumulation of p65/RelA subunit of NF- κ B was studied (Fig. 5B and C). These experiments suggest that IKK α may make a minor contribution to the nuclear accumulation of IRF5 or p65/RelA in Gen2.2 cells, if IKK β is inhibited. The knockdown of TAK1, also abolished the nuclear accumulation of IRF5 (Fig. 5D). This is as expected because TAK1 is required for the activation of IKK α and IKK β .

The TLR7-Stimulated Nuclear Translocation of IRF5 at Ser462 in RAW264.7 Cells Requires IKK β Activity. IRF5 is required for the production of proinflammatory cytokines, such as IL-12 and TNF in macrophages and conventional dendritic cells (1). We found that the TLR7 agonist R848 stimulated the nuclear translocation of IRF5-GFP, but not the IRF5[S462A]-GFP mutant, in the murine RAW264.7 macrophage-like cell line (Fig. 3B and Fig. S3C). Moreover, the IKK β inhibitors BI605906 and PS1145 or

the TAK1 inhibitor NG-25 prevented the nuclear translocation of IRF5-GFP, whereas the TBK1/IKK ϵ inhibitor MRT67307 (Fig. 4B and Fig. S3D), the p38 MAP kinase inhibitor compound BIRB0796 (21), the mitogen-activated protein kinase or extracellular signal regulated kinase 1/2 (MEK1/2) inhibitor compound PD0325901 (22), or the combination of both BIRB0796 and PD0325901 (Fig. S5A) did not. In control experiments BIRB0796 blocked the phosphorylation of MAPKAP kinase-2 (MK2), a substrate of p38 α MAP kinase, whereas PD0325901 suppressed the phosphorylation of extracellular signal regulated kinases 1 and 2 (ERK1 and ERK2), as expected. The phosphorylation of mitogen- and stress-activated kinases 1 and 2 (MSK1/MSK2), which is catalyzed by both p38 α MAP kinase and ERK1/2 (23), was prevented by a combination of BIRB0796 and PD0325901 (Fig. S5B).

Discussion

In this study, we have established that the IKK β -catalyzed phosphorylation of Ser462 is required for its dimerization and nuclear translocation of IRF5 by ligands that activate TLR7 in myeloid cells. The mutation of Ser462 to Ala or the suppression of Ser462 phosphorylation with inhibitors of the activity or activation of IKK β blocked the nuclear entry of IRF5 in response to ligands that activate TLR7. The results provide a molecular explanation for our earlier finding that the TLR7-stimulated

Fig. 5. The nuclear translocation of the endogenous IRF5 is prevented by the inhibition or knockdown of IKK β or TAK1 in Gen2.2 cells. (A) Gen2.2 cells were incubated for 1 h without (–) or with (+) NG-25 (2.0 μ M), BI605906 (5.0 μ M), PS1145 (15 μ M), or MRT67307 (2.0 μ M), then stimulated for 30 min with CL097 (1.0 μ g/mL). Nuclei were separated from the cytosol, lysed, and the localization of IRF5 and p65/RelA analyzed by immunoblotting. GADPH and SMC-1 were used as loading controls for the cytosol and nuclear extracts, respectively. (B–D) Gen2.2 cells were transfected with control siRNA or siRNA for IKK β (B), IKK α (C), or TAK1 (D). After 72 h, the cells were treated as in A. The cell extracts prepared before fractionation into nucleus and cytosol were immunoblotted with antibodies that recognize p38 α MAP kinase (as a loading control), a phospho-specific antibody that recognizes the IKK substrate p65, and with either IKK β (B), IKK α (C), or TAK1 (D). All of the experiments in A–D were repeated at least three times with similar results.

production of IFN β in the human pDC line Gen2.2 is dependent on IKK β activity, but independent of the activation of NF- κ B (18). Ser462 of IRF5 is conserved in all vertebrate orthologs of this protein (Fig. 6A). Interestingly, this serine is situated at a position equivalent to Ser396 of IRF3 (Fig. 6B), whose phosphorylation by TBK1 is important for the nuclear translocation of IRF3. Ser462 of IRF5 is positioned within hydrogen-bonding distance of Arg354, an arginine residue that is conserved in IRF3 (24). The phosphorylation of Ser462 of IRF5 or Ser396 of IRF3 may therefore induce an interaction between the phosphoserine and arginine residues that stabilizes the dimeric form of IRF5 and permits its nuclear translocation and the stimulation of gene transcription. Consistent with this notion, we observed that IKK β induced the dimerization of wild-type IRF5, but not the IRF5[S462A] mutant in cotransfection experiments, and that only the dimeric form was phosphorylated at Ser462 (Fig. 2D). The IKK β -catalyzed phosphorylation of IRF5 at Ser462 may underlie the activation of this transcription factor by many agonists, because compound KF-1B, a ligand that activates the cytosolic NOD1 receptor, also stimulated the nuclear translocation of IRF-GFP, but not the IRF5[S462A]-GFP mutant in RAW264.7 cells, and translocation was suppressed by the inhibition of IKK β or TAK1, but not by the inhibition of TBK1/IKK ϵ (Fig. S6).

Ser462 was the only phosphorylation site in the endogenous IRF5 that we detected in 10 independent SILAC-mass spectrometry experiments performed in TLR7-stimulated Gen2.2 cells. In contrast, other investigators identified six phosphorylation sites when IRF5 was overexpressed in HEK293 cells with either TBK1, TRAF6, or Receptor-Interacting Protein Kinase 2 (RIPK2) (16). Cotransfection with TBK1 induced the phosphorylation of IRF5 at Ser158 and Ser309, whereas

four other sites (Thr10, Ser317, Ser451, and Ser462) were phosphorylated after cotransfection with TRAF6 or RIPK2. Mutagenesis studies indicated that Ser462 was the most critical phosphorylation needed for IL-12p40 gene transcription. A further reduction in IL-12p40 gene transcription occurred when both Ser451 and Ser462 were mutated to Ala. Conversely, the mutation of Ser462 and Ser451 to Asp (to mimic the effect of phosphorylation by introducing a negative charge) induced both nuclear translocation and IL-12p40 reporter gene expression (16). These studies are consistent with an important role for Ser462 phosphorylation in permitting IRF5 to undergo nuclear translocation and stimulate IL-12p40 gene transcription (16).

The expression of RIPK2 is essential for activation of the NOD1/2 signaling network and can activate NF- κ B in overexpression experiments. However, its kinase activity is not required for activation and a catalytically inactive mutant is even more efficient than wild-type RIPK2 in inducing NF- κ B and MAP kinase activation (25). The overexpression of TRAF6 can also activate NF- κ B (e.g., ref. 26). However, our results suggest that cell transfection with RIPK2 or TRAF6 induces IRF5 phosphorylation at Ser462 indirectly by activating IKK β .

TBK1 has been implicated in the TLR7-stimulated activation of IRF5 (9), but we found that the nuclear translocation of IRF5 was unaffected by MRT67307, a potent inhibitor of TBK1 and the related kinase IKK ϵ (Figs. 4 and 5 and Fig. S3B and D). Moreover, TBK1 does not phosphorylate IRF5 at Ser462 in vitro (Fig. 2C) and we have failed so far to detect phosphorylation of the endogenous IRF5 at Ser158 and Ser309 that are targeted by TBK1 in vitro. Therefore, whether phosphorylation of the serine residues that can be forced by overexpression with TBK1 (16) has physiological relevance, is unclear.

In summary, our results establish that IKK β activates two "master" transcription factors of the innate immune system, IRF5 and NF- κ B (Fig. 6C). The phosphorylation induces the nuclear translocation of these proteins stimulating the transcription of IL-12 and other inflammatory cytokines in macrophages and IFN β in pDCs. Further research is required to understand how IKK β mediates the TLR9-stimulated production of IFN α (18) and why IKK α is needed for IFN production in pDCs (18, 27).

Materials and Methods

Agonists and Inhibitors. The TAK1 inhibitor NG-25 (28), the IKK β inhibitor BI605906 (20), KF-1B (29, 30), MRT67307 (31), BIRB0796 (32), and PD0325901 (33) were synthesized as described. The TLR7 agonists, CL097 (catalog no. tlr1-c97) and R848 (tlr1-r848) were purchased from Invivogen. LPS (lipopolysaccharide; *Escherichia coli* 055:B5) was from Alexis Biochemicals (ALX-581-001) and PS1145 (34) from Sigma.

Immunofluorescence Studies. The Gen2.2 cells are difficult to transfect with cDNA and several transfection protocols tested did not result in any transfection. The successful procedure, which consistently produced transfection of 10% of the cells is outlined below. The 2×10^6 Gen2.2 cells were nucleofected with 1.0 μ g IRF5-GFP or IRF5[S462A]-GFP cDNA using the Amaxa nucleofector, A-033 or X-001 programs. After 24 h, cells were replated at a density of 10^6 cells/mL. After 24 h, cells were incubated for 60 min with inhibitors and then stimulated with ligands, as specified in the figure legends. The cells were fixed for 10 min with 4% (vol/vol) formaldehyde and 50,000 cells were centrifuged into precoated slides (Thermo Scientific). The cells were permeabilized by incubation for 10 min with methanol at -20°C . RAW264.7 cells were treated similarly, except that 2.0 μ g IRF5-GFP or IRF5[S462A]-GFP cDNA were nucleofected using the Amaxa nucleofector D-032 program. After 24 h, 10^5 cells were plated on coverslips, inhibitors were added after 48 h rather than 24 h, and after fixing in formaldehyde, the cells were permeabilized for 10 min with 0.2% (vol/vol) Triton X-100.

The Gen2.2 and RAW264.7 cells were incubated for 1 h with 2% (wt/vol) BSA, incubated for 16 h at 4°C with anti-GFP (Abcam 1:2,000),

Fig. 6. IKK β activates IRF5 by phosphorylating Ser462. (A) Ser462 of IRF5 is conserved during vertebrate evolution. Identical amino acid residues are shown in white letters on a black background and conservative replacements in black letters on a gray background. (B) Ser462 of IRF5 lies in an equivalent position to Ser396 of IRF3. (C) IKK β phosphorylates the transcription factors IRF5 and NF- κ B inducing their nuclear translocation where they stimulate gene transcription. IKK β phosphorylates the I κ B α subunit of NF- κ B, which leads to its ubiquitination and degradation by the proteasome, and the p65/RelA component at two or more sites (20). The p65/RelA enters the nucleus as a complex with p50.

washed with 0.2% (vol/vol) Tween in PBS at ambient temperature, incubated for 1 h at 21 °C with the secondary antibody Alexa 448 (Life Technologies 1:5,000), and counterstained with DAPI (0.2 µg/mL) to reveal nuclei. Images were acquired using a Delta Vision DV3 deconvolution microscope with an immersion-oil 63× objective lens and images were processed using OMERO. Images presented correspond to one tack from deconvolved 3D images.

All other materials and methods are described in *SI Materials and Methods*.

1. Takaoka A, et al. (2005) Integral role of IRF-5 in the gene induction programme activated by Toll-like receptors. *Nature* 434(7030):243–249.
2. Yasuda K, et al. (2007) Murine dendritic cell type I IFN production induced by human IgG-RNA immune complexes is IFN regulatory factor (IRF)5 and IRF7 dependent and is required for IL-6 production. *J Immunol* 178(11):6876–6885.
3. Purtha WE, Swiecki M, Colonna M, Diamond MS, Bhattacharya D (2012) Spontaneous mutation of the Dock2 gene in Irf5^{-/-} mice complicates interpretation of type I interferon production and antibody responses. *Proc Natl Acad Sci USA* 109(15):E898–E904.
4. Yasuda K, et al. (2013) Phenotype and function of B cells and dendritic cells from interferon regulatory factor 5-deficient mice with and without a mutation in DOCK2. *Int Immunol* 25(5):295–306.
5. Steinhagen F, et al. (2013) IRF-5 and NF-κB p50 co-regulate IFN-β and IL-6 expression in TLR9-stimulated human plasmacytoid dendritic cells. *Eur J Immunol* 43(7):1896–1906.
6. Gratz N, et al. (2011) Type I interferon production induced by Streptococcus pyogenes-derived nucleic acids is required for host protection. *PLoS Pathog* 7(5):e1001345.
7. del Fresno C, et al. (2013) Interferon-β production via Dectin-1-Syk-IRF5 signaling in dendritic cells is crucial for immunity to *C. albicans*. *Immunity* 38(6):1176–1186.
8. Barnes BJ, Kellum MJ, Field AE, Pitha PM (2002) Multiple regulatory domains of IRF-5 control activation, cellular localization, and induction of chemokines that mediate recruitment of T lymphocytes. *Mol Cell Biol* 22(16):5721–5740.
9. Schoenemeyer A, et al. (2005) The interferon regulatory factor, IRF5, is a central mediator of toll-like receptor 7 signaling. *J Biol Chem* 280(17):17005–17012.
10. Barnes BJ, Moore PA, Pitha PM (2001) Virus-specific activation of a novel interferon regulatory factor, IRF-5, results in the induction of distinct interferon alpha genes. *J Biol Chem* 276(26):23382–23390.
11. Barnes BJ, et al. (2004) Global and distinct targets of IRF-5 and IRF-7 during innate response to viral infection. *J Biol Chem* 279(43):45194–45207.
12. Lin R, Yang L, Arguello M, Penafuerte C, Hiscott J (2005) A CRM1-dependent nuclear export pathway is involved in the regulation of IRF-5 subcellular localization. *J Biol Chem* 280(4):3088–3095.
13. Fitzgerald KA, et al. (2003) IKKε and TBK1 are essential components of the IRF3 signaling pathway. *Nat Immunol* 4(5):491–496.
14. Hemmi H, et al. (2004) The roles of two IκappaB kinase-related kinases in lipopolysaccharide and double stranded RNA signaling and viral infection. *J Exp Med* 199(12):1641–1650.
15. McWhirter SM, et al. (2004) IFN-regulatory factor 3-dependent gene expression is defective in Tbk1-deficient mouse embryonic fibroblasts. *Proc Natl Acad Sci USA* 101(1):233–238.
16. Chang Foreman HC, Van Scoy S, Cheng TF, Reich NC (2012) Activation of interferon regulatory factor 5 by site specific phosphorylation. *PLoS ONE* 7(3):e33098.
17. Gilliet M, Cao W, Liu YJ (2008) Plasmacytoid dendritic cells: Sensing nucleic acids in viral infection and autoimmune diseases. *Nat Rev Immunol* 8(8):594–606.
18. Pauls E, et al. (2012) Essential role for IKKβ in production of type 1 interferons by plasmacytoid dendritic cells. *J Biol Chem* 287(23):19216–19228.
19. Barnes BJ, Field AE, Pitha-Rowe PM (2003) Virus-induced heterodimer formation between IRF-5 and IRF-7 modulates assembly of the IFNA enhanceosome in vivo and transcriptional activity of IFNA genes. *J Biol Chem* 278(19):16630–16641.
20. Clark K, et al. (2011) Novel cross-talk within the IKK family controls innate immunity. *Biochem J* 434(1):93–104.
21. Kuma Y, et al. (2005) BIRB796 inhibits all p38 MAPK isoforms in vitro and in vivo. *J Biol Chem* 280(20):19472–19479.
22. Bain J, et al. (2007) The selectivity of protein kinase inhibitors: A further update. *Biochem J* 408(3):297–315.
23. Wiggan GR, et al. (2002) MSK1 and MSK2 are required for the mitogen- and stress-induced phosphorylation of CREB and ATF1 in fibroblasts. *Mol Cell Biol* 22(8):2871–2881.
24. Chen W, et al. (2008) Insights into interferon regulatory factor activation from the crystal structure of dimeric IRF5. *Nat Struct Mol Biol* 15(11):1213–1220.
25. Windheim M, Lang C, Pegg M, Plater LA, Cohen P (2007) Molecular mechanisms involved in the regulation of cytokine production by muramyl dipeptide. *Biochem J* 404(2):179–190.
26. Starczynowski DT, et al. (2011) TRAF6 is an amplified oncogene bridging the RAS and NF-κB pathways in human lung cancer. *J Clin Invest* 121(10):4095–4105.
27. Hoshino K, et al. (2006) IκappaB kinase-α is critical for interferon-α production induced by Toll-like receptors 7 and 9. *Nature* 440(7086):949–953.
28. Tan L, et al. (2014) Discovery of type II inhibitors of TGFβ-activated kinase 1 (TAK1) and mitogen-activated protein kinase kinase kinase 2 (MAP4K2). *J Med Chem*, 10.1021/jm500480k.
29. Hasegawa M, et al. (2007) A role of lipophilic peptidoglycan-related molecules in induction of Nod1-mediated immune responses. *J Biol Chem* 282(16):11757–11764.
30. Masumoto J, et al. (2006) Nod1 acts as an intracellular receptor to stimulate chemokine production and neutrophil recruitment in vivo. *J Exp Med* 203(1):203–213.
31. McIver EG, et al. (2012) Synthesis and structure-activity relationships of a novel series of pyrimidines as potent inhibitors of TBK1/IKKε kinases. *Bioorg Med Chem Lett* 22(23):7169–7173.
32. Regan J, et al. (2002) Pyrazole urea-based inhibitors of p38 MAP kinase: From lead compound to clinical candidate. *J Med Chem* 45(14):2994–3008.
33. Barrett SD, Biwersi C, Kaufman M, Tecle H, Warmus JS (2002) Preparation of oxygenated esters of 4-iodophenylaminobenzhydroxyamic acids as MEK inhibitors. *World Pat. WO/2002/006213*.
34. Castro AC, et al. (2003) Novel IKK inhibitors: Beta-carbolines. *Bioorg Med Chem Lett* 13(14):2419–2422.
35. Ren J, Chen X, Chen ZJ (2014) IKKβ is an IRF5 kinase that instigates inflammation. *Proc Natl Acad Sci USA* 111:17438–17443.