A Role for Mixed Lineage Kinases in Regulating Transcription Factor CCAAT/Enhancer-binding protein-β-dependent Gene Expression in Response to Interferon-γ*

Sanjit K. Roy†, Jon D. Shuman§, Leonidas C. Plataniaς, Paul S. Shapiro*, Sekhar P. M. Reddy**, Peter F. Johnson§, and Dhananjaya V. Kalvakolanu‡ ‡‡

From the †Greenebaum Cancer Center, Department of Microbiology and Immunology, and Molecular and Cellular Biology Program, University of Maryland School of Medicine and the School of Pharmacy, Baltimore, Maryland 21201, the §Laboratory of Protein Dynamics and Signaling, NCI, National Institutes of Health, Frederick, Maryland 21702, the *Robert Lurie Cancer Center, Northwestern University School of Medicine, Chicago, Illinois 60611, and the ‡‡Department of Environmental Health Sciences, Bloomberg School of Public Health, The Johns Hopkins University, Baltimore, Maryland 21205.

Transcription factor CCAAT/enhancer-binding protein-β (C/EBP-β) regulates a variety of cellular functions in response to exogenous stimuli. We have reported earlier that C/EBP-β induces gene transcription through a novel interferon (IFN)-response element called γ-IFN-activated transcriptional element. We show here that IFN-γ-induced, C/EBP-β/γ-IFN-activated transcriptional element-dependent gene regulation is expressed by mixed lineage kinases (MLKs), members of the mitogen-activated protein kinase kinase family. MLK3 appears to activate C/EBP-β in response to IFN-γ by a mechanism involving decreased phosphorylation of a specific phosphoacceptor residue, Ser64, within the transactivation domain. Decreased phosphorylation of Ser64 was independent of IFN-γ-stimulated ERK1/2 activation and did not require the ERK phosphorylation site Thr189, located in regulatory domain 2 of C/EBP-β. Together these studies provide the first evidence that MLK3 is involved in IFN-γ signaling and identify a novel mechanism of transcriptional activation by IFN-γ.

Interferons regulate the antiviral, antitumor, and immune responses by inducing the transcription of a number of IFN-γ-stimulated genes (ISGs). IFN-γ regulates a number of diverse processes including cell growth and innate and specific immune responses (1). Although STAT1 is a critical primary regulator of IFN-γ-induced responses, several ISGs are critically dependent on other transcription factors. A STAT1-independent regulation of some genes by IFN-γ has been shown (2, 3). irf9 is an IFN-γ inducible gene that codes for a DNA-binding protein. In the IFN-α signal transduction pathway the IRF9 protein forms a trimeric transcription factor, ISGF3, in association with the STAT1 and STAT2 proteins to drive gene expression (4, 5). IFN-γ potently induces the expression of IRF9 to augment IFN-α/β-induced transcription (6, 7). Indeed down-regulation of irf9 expression by viral products provides an escape route against the antiviral action of IFNs (8). Interestingly the irf9 promoter lacks STAT-binding elements (9). Instead a novel regulatory element (termed GATE) and its cognate transcription factors control irf9 expression. We have shown earlier that the bZIP transcription factor C/EBP-β, a regulator of acute phase responses and cell differentiation (10, 11), binds to GATE and induces IFN-regulated transcription (12). C/EBP-β (nuclear factor induced by interleukin-6, liver-activated protein, C/EBP-related protein 2, nuclear factor myeloid) (10, 11, 13) responds to a number of extracellular stimuli including interleukin-6, interleukin-1, tumor necrosis factor-α, and lipopolysaccharide (10, 11) and is necessary for regulating several processes including carbohydrate metabolism, lipid storage, Th1 immune responses, macrophage-mediated antibacterial and antitumor defenses, and female fertility (11, 13–15). C/EBP-β deficiency causes a lymphoproliferative disorder (16), and C/EBP-β is also required for B-lymphopoiesis (17), chemical carcinogen-induced Ras-dependent tumor formation in skin keratinocytes (18), and growth factor-independent survival of Myc/Raf-transformed macrophage tumor cells (19).

Our recent studies have shown that mitogen-activated protein kinases (MAPKs) play a critical role in regulating C/EBP-β and GATE-dependent gene expression. This occurs at least in part by phosphorylation of C/EBP-β on Thr189 by ERK1/2 (20, 21). Here we show a role for mixed lineage kinases (MLKs), a subgroup of upstream kinases that regulate the MAPK family, in controlling C/EBP-β-dependent transcription. MLKs function as MAPK kinase kinases and have been implicated in the activation of c-Jun N-terminal kinase (JNK) and stress-activated protein kinase kinase 1 and transcription factor NF-κB. Some MLKs also activate p38 MAPK. Interestingly instead of stimulating phosphorylation, MLKs exerted a posi-

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‡‡ To whom correspondence should be addressed: Greenebaum Cancer Center, 665 W. Baltimore St., 9th floor, Baltimore, MD 21201. Tel.: 410-328-1396; E-mail: dkalvako@umaryland.edu.

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tive effect on the transactivation domain of C/EBP-β by promoting decreased phosphorylation at a specific serine residue in the transactivation domain. Thus, our studies identified a novel IFN-γ effector pathway involving MLKs and C/EBPβ.

MATERIALS AND METHODS

Reagents—Murine IFN-γ (Pestka Biomedical Laboratories); SB202190 (Calbiochem); antibodies specific for phospho-ERK1/2; actin (Sigma); native ERK2; and hemagglutinin (HA) epitope tag, transcriptional co-activator p300, and C/EBP-β (Santa Cruz Biotechnology) were used in these studies. MLK inhibitor CEP-11004 (henceforth referred to as CEP) was generously provided by Cephalon Inc., West Chester, PA. An antibody that detects a phospho-Thr189 form (Thr(P)189) of C/EBP-β was purchased from Cell Signaling Technology Inc. An antibody that specifically detects the phospho-Ser64 form (Ser(P)64) of C/EBP-β has been described elsewhere (22). Affinity-purified rabbit polyclonal antibodies specific for the native (Santa Cruz Biotechnology) and activated (BIOSOURCE) forms of MLK3 were used in some experiments. In response to extracellular stimuli, the two critical threonine (Thr277) and serine (Ser280) residues located in the activation loop of MLK3 are phosphorylated (23). The antibody against the activated MLK3 isoform detects the diphosphorylated (Thr(P)277/Ser(P)280) form of the MLK3 protein.

Cell Culture and Plasmids—The murine macrophage cell line RAW (RAW264.7) was grown in RPMI 1640 medium with 5% fetal bovine serum. Isogenic mouse embryonic fibroblasts (MEFs) derived from wild type, mekk1−/− (24) and cebpβ−/− (21) mice were described earlier. These cells were cultured in Dulbecco’s modified Eagle’s medium with 2% fetal bovine serum during IFN-γ treatment. The murine ISGF3γ (p48) reporter construct, P4, was described earlier (9). In this construct a 74-bp element of murine p48 gene promoter, encompassing GATE, was cloned upstream of the SV40 early promoter driving luciferase. Mutagenesis of the GATE sequence in this construct caused a loss of IFN-γ response and C/EBP-β binding (12). A wild type and a catalytically inactive dominant negative mutant (K114R) of MLK3 cloned in pcDNA3.0 vector was described earlier (25). The native irf9 promoter (pIRE) and the corresponding GATE mutant were described earlier (9). A mammalian expression vector with the transactivation domain (TAD) (amino acids 22–109) of C/EBP-β and its corresponding S64A mutant fused to the DNA binding domain of transcription factor GAL4 were described previously (26). The G-E1b-Luc reporter contains five tandem copies of the GAL4 binding sites placed upstream of minimal EF-1α promoter-driven pIRE-Luc reporter (0.2 ng). The total amount of transfected DNA (1.0 μg) was kept constant by adding pBluescript SK DNA if required. In general, 0.4 μg of luciferase and 0.1 μg of C/EBP-β expression vector were used for transfection. A β-actin promoter-driven β-galactosidase reporter (0.2 μg) was used as an internal control for normalizing variations in transfection efficiency. ERK activation was monitored by Western blot analysis of cell extracts with a phospho-ERK1/2-specific antibody (Cell Signaling Technology Inc.). Anti-ERK2 antibodies were used for determining the total ERK in these samples. In some experiments antibodies specific for the native and phosphorylated forms of MLK3 were used.

Chromatin Immunoprecipitation Assays—These assays were performed as described earlier (29). Briefly cells (1 × 10⁵) were stimulated with IFN-γ for 8 h, and chromatin was cross-linked using paraformaldehyde. Nuclei were isolated, and chromatin was sheared into ~ 1–2-kb fragments using a Bronson sonicator fitted with a microtip probe. After removing the debris, soluble chromatin was subjected to IP with either

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**Fig. 1. Effect of CEP on IFN-γ-induced gene expression. RAW macrophages were transfected with various luciferase reporter constructs (200 ng) and pCMV-β-galactosidase (50 ng) and treated with murine IFN-γ (500 units/ml) and/or CEP (160 nm) for 16 h. A plus sign (+) indicates treatment with the indicated agent. Luciferase activity was determined and normalized to that of β-galactosidase activity and presented as relative light units (RLU) ± S.E. Reporters used in each panel are: A, P4-luciferase reporter; B, open bars, the native irf9 promoter; filled bars, native irf9 promoter with a mutated GATE; C, pIRE-Luc; D, AP1RE-Luc. In D epidermal growth factor (EGF) (10 ng/ml) was used as an inducer.**

C/EBP-β- or transcriptional co-activator p300-specific antibodies. After extensively washing the immunoprecipitated products, cross-links were reversed, and the DNA was extracted using phenol-chloroform. The resultant DNA was used for 32 cycles of PCR with the following primers specific for mouse irf9 promoter: 5'-AAGGTGCTACTGCTGACTGAGG-3' and 5'-AAGGGCCGACGTGAAAGAAATG-3'. PCR with these primers yields a 443-bp product. DNA extracted from an aliquot of the initial soluble chromatin was used for input control.

**RESULTS**

IFN-γ-induced Gene Expression through GATE Is Inhibited by MLK Inhibitor CEP-11004—To examine the role of MLKs in IFN-γ-regulated signaling pathways, we used a specific semi-synthetic inhibitor, CEP. A previously defined concentration of CEP that specifically inhibited MLKs (30), but not other MAPks, was used in this study. These experiments were performed using the murine macrophage cell line RAW264.7. Cells were transfected with the P4 reporter gene and then treated with the inhibitors prior to IFN-γ treatment. CEP strongly inhibited (Fig. 1A) GATE-driven transcription. Under these conditions, SB202190, a pan-p38 MAPK-specific inhibitor, did not inhibit IFN-γ-induced gene expression through GATE (data not shown). Similar to P4-Luc, expression of Δ6-Luc, a reporter bearing the native irf9 promoter but not its corresponding mutant lacking a functional GATE, was induced following IFN-γ treatment (Fig. 1B). Such induction was inhibited by CEP. Thus, the native IRF9 enhancer and minimal enhancer were sensitive to CEP inhibition. To determine whether CEP exerted a similar inhibitory effect on another IFN-γ-regulated enhancer element we used palindromic IFN-response element (pIRE)-luciferase, whose expression is dependent on IFN-γ-activated STAT1 binding to the pIRE-STAT1-dependent expression of pIRE-Luc was unaffected by CEP (Fig. 1C). Similarly CEP did not significantly inhibit epidermal growth factor-induced expression through an activator protein 1-responsive element (Fig. 1D). Together these results suggest that MLK activity is critical for IFN-γ-dependent induction of IRF9.

To test whether CEP exhibited a similar inhibitory effect on the expression of the endogenous irf9 gene, we performed reverse transcription-PCR analysis of irf9 mRNA levels following various treatments (Fig. 2A). CEP strongly inhibited the IFN-γ-induced expression of irf9, whereas it had no significant effect on the basal expression of the gene. In contrast, expression of another IFN-γ-regulated mRNA, IRF8/IFN consensus
GATE-dependent transcription. 

Together with data in Figs. 1 and 2, these observations indicate expression is inhibited by CEP. However, they did not identify the above studies indicate that GATE-driven gene post-translational modification(s) of C/EBP-

sequence-binding protein, was not inhibited by CEP (Fig. 2B). This gene is dependent on STAT1 for its expression. The expression of glyceraldehyde-3-phosphate dehydrogenase mRNA was not affected by any of the treatments (Fig. 2C). These results demonstrate a specific inhibitory effect of CEP on a subset of IFN-induced genes.

**CEP Inhibits C/EBP-β-dependent Gene Expression through GATE**—The above studies indicate that GATE-driven gene expression is inhibited by CEP. However, they did not identify the transcription factor that is subject to inhibition. Our previous studies have established C/EBP-β as a critical regulator of IFN-γ-induced gene expression through GATE (12). To demonstrate that C/EBP-β is indeed the target of CEP inhibition, we performed a transient transfection assay with the P4 reporter in cebpb−/− MEFs in the presence of exogenous C/EBP-β. Co-transfection of C/EBP-β but not an empty vector conferred a strong induction of luciferase (Fig. 3A). As reported earlier, C/EBP-β also induced the basal expression of this promoter (12). CEP potently inhibited C/EBP-β-dependent IFN-γ-induced expression of the reporter but did not affect basal expression. These differential effects on the promoter are not due to variable expression of the C/EBP-β protein because a comparable level of C/EBP-β protein was found between the samples (Fig. 3B). These data suggest that an IFN-γ-induced post-translational modification(s) of C/EBP-β may be inhibited by CEP.

Because CEP is an inhibitor of the mixed lineage kinases (30), we next determined whether a dominant negative MLK3 inhibits GATE-dependent transcription. We chose to focus on MLK3 because it is a ubiquitously expressed enzyme and has been relatively well characterized compared with the other known members of its family (23). Vectors carrying wild type Mlk3 and a catalytically inactive mutant were co-expressed with the P4-luciferase reporter to determine their effects on IFN-γ-induced expression through GATE (Fig. 3D). Wild type Mlk3 significantly augmented the IFN-γ-stimulated expression of the reporter (3-4-fold), whereas the catalytically inactive mutant significantly (3-fold) inhibited it. Neither wild type nor mutant Mlk3 altered the basal expression of the reporter. Together with data in Figs. 1 and 2, these observations indicate that MLK3 plays an important role in regulating IFN-induced GATE-dependent transcription.

We next checked the relevance of endogenous Mlk3 to IFN-γ-induced gene expression through GATE using siRNAs. Because the mouse and human MLK3 siRNA targets differ in their primary sequence they degrade their target mRNAs in a species-specific manner (31). We first examined the specificities of these siRNAs in knocking down the expression of Mlk3. RAW cells were transfected with the pSuppressor-Neo vector expressing mouse or human MLK3-specific siRNAs. The mouse Mlk3 siRNA knocked down the expression of MLK3 protein (70%) compared with the empty vector control. As expected, the human MLK3 siRNA did not affect the MLK3 expression (Fig. 3F). A converse effect of mouse siRNA on the expression of human MLK3 was found in HeLa cells (data not shown).

Having demonstrated the specificity of these siRNAs, we next tested their effects on IFN-γ-induced transcription in RAW cells. These siRNA vectors were co-transfected with two IFN-γ-inducible reporters: 1) P4-Luc and 2) pIRE-Luc. As mentioned earlier the former is dependent on C/EBP-β and the latter is dependent on STAT1 for their expression. As shown in Fig. 3G, co-expression of mouse MLK3 siRNA, but not the human MLK3 siRNA, significantly inhibited the IFN-γ-induced expression of the P4-Luc compared with vector control. In contrast, these siRNAs did not affect expression of the pIRE-Luc. These data clearly indicate an important role for MLK3 in IFN-γ-induced transcription driven by GATE (Fig. 3G).

The MLK proteins are expressed in a tissue-specific manner (23). MLK3 protein is expressed ubiquitously. The irf9 gene is induced by IFN-γ in most cell types, consistent with the ubiquitous expression of MLK3. In contrast, the expression of MLK2 is restricted to skeletal muscle, brain, and testis. To test the specificity of MLKs, we examined whether co-expression of MLK2 would also augment IFN-γ-induced transcription. For this purpose a GFP-tagged MLK2 protein was co-expressed with the P4-Luc reporter. GFP-MLK2 did not significantly augment the IFN-γ-induced expression of the luciferase gene when compared with the control, GFP expression vector alone (Fig. 3H). Expression of GFP-tagged MK2 was comparable in the control and IFN-γ-treated cells (Fig. 3I).

**IFN-γ Activates MLK3**—To address the issue of whether endogenous MLK3 is activated, we stimulated RAW cells with IFN-γ for various lengths of time and prepared the cellular lysates. The protein extracts were subjected to Western blot analyses with antibodies that can specifically detect the native and diphosphorylated (Thr277/Ser281) forms of MLK3. Like the other members of the MAPK family, MLK3 is activated by phosphorylation at two critical threonine and serine (Thr277/Ser281) residues present in the activation loop (23). Following phosphorylation, MLK3 gains its catalytic activity. These analyses showed a time-dependent increase in MLK3 phosphorylation following treatment with IFN-γ (Fig. 4A). As early as 30 min after stimulation with IFN-γ a significant rise in MLK3 phosphorylation occurred compared with the control. Such activation continued to increase up to 6 h and declined thereafter. The kinetics of MLK3 activation is consistent slow activation of the irf9 gene in response to IFN-γ. We next tested whether CEP blocked the IFN-γ-induced activation of MLK3 using the phosphospecific antibodies described above (Fig. 4B). Cells were exposed to IFN-γ in the absence and presence of CEP. As expected, IFN-γ induced the activation of MLK3 protein. Incubation of cells with CEP blocked such activation. These differences in the activation of MLK3 are not due to different levels of MLK3 protein. We could not measure the activation of other MLKs for the following reasons. 1) Unlike MLK3, the expression of MLK2 is restricted to very few tissues, and 2) no native- or activation-specific antibodies are available for the other isoforms.

**CEP Does Not Inhibit the ERK-induced Phosphorylation at Thr189 of C/EBP-β**—We have shown earlier that ERK1/2 signals play an important role in regulating the irf9 gene (20). Therefore, we examined whether CEP interfered with ERK1/2 activation in response to IFN-γ. Western blot analysis with antibodies specific for diphosphorylated forms of ERK1/2

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**Fig. 2.** Effect of CEP on the expression of endogenous IFN-γ-regulated genes. RAW cells were treated with IFN-γ for 8 h in the absence and presence of CEP. RNA was extracted, and reverse transcription-PCR was performed with gene-specific primers. A, IRF9, a C/EBP-β-regulated gene; B, IRF8, a STAT1-regulated gene; C, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). D shows the relative induction of IRF8 and IRF9 mRNAs.

**Fig. 3.** A, IFN-γ induced the activation of IRF9 and IRF8, a STAT1-regulated gene, D, GAPDH as a control for gene expression. B, Western blot analysis with antibodies specific for diphosphorylated forms of ERK1/2 phosphorylation following treatment with IFN-γ (Fig. 4A). As early as 30 min after stimulation with IFN-γ a significant rise in MLK3 phosphorylation occurred compared with the control. Such activation continued to increase up to 6 h and declined thereafter. The kinetics of MLK3 activation is consistent with slow activation of the irf9 gene in response to IFN-γ. We next tested whether CEP blocked the IFN-γ-induced activation of MLK3 using the phosphospecific antibodies described above (Fig. 4B). Cells were exposed to IFN-γ in the absence and presence of CEP. As expected, IFN-γ induced the activation of MLK3 protein. Incubation of cells with CEP blocked such activation. These differences in the activation of MLK3 are not due to different levels of MLK3 protein. We could not measure the activation of other MLKs for the following reasons. 1) Unlike MLK3, the expression of MLK2 is restricted to very few tissues, and 2) no native- or activation-specific antibodies are available for the other isoforms.

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showed that ERK1/2 were activated equivalently by IFN-γ/H9253 in the presence and absence of CEP (Fig. 5A).

**Analysis of total ERK2 levels revealed a comparable expression (Fig. 5B).** Thus, CEP did not inhibit ERK activation by IFN-γ/H9253.

Because our earlier studies identified an important role for the Thr189 residue (a target for ERK1/2-induced phosphorylation), located in the regulatory domain 2 of C/EBP-H9253, in IFN-γ/H9253-induced gene expression (20, 21), we next examined whether CEP affected the IFN-γ/H9253-induced phosphorylation of C/EBP-H9253 at Thr189. An antibody that detects the phospho-Thr189 form of C/EBP-H9253 was used as a tool for detecting changes in ligand-induced phosphorylation. The specificity of anti-Thr(P)189 antibody is shown in Fig. 5C.

In this experiment protein lysates from cebp−/− cells transfected with wild type (188GTPS191) and a corresponding alanine-substituted mutant (188GAAA191) were used for Western blot analysis (B and C) and probed with C/EBP-β- and actin-specific antibodies. D, MLK3 promotes C/EBP-β-dependent gene expression in response to IFN-γ. RAW cells were transfected with expression vectors (100 ng) coding for HA-tagged wild type or catalytically inactive MLK3 proteins along with the P4-Luc reporter (200 ng). Luciferase activity was determined after various treatments.

**E, expression of the MLK3 proteins was monitored using HA tag-specific antibodies. F, effect of MLK3 siRNAs on MLK3 expression.** Cell lysates were prepared after 36 h of transfection of the pSuppressor-Neo (pSup-Neo, empty expression vector) or the same expression vector coding for mouse (mMLK3 siRNA) and human (hMLK3 siRNA) siRNAs specific for MLK3. A comparable amount of the total protein (100 μg) was Western blotted with MLK3- and actin-specific antibodies.

**G, effect of MLK3 siRNAs on the IFN-γ-induced expression of P4-Luc and pIRE-Luc.** RAW cells were transfected with equal quantities (100 ng) of reporter gene and the siRNA expression vectors. The cells were also transfected with 50 ng of pCMV-β-galactosidase reporter for normalizing variations in transfection efficiency. Normalized luciferase activities (as relative light units (RLU)) are shown. **H, effect of MLK2 on the IFN-γ-induced expression of P4-Luc.** Cells were transfected with the pEGFP-N1 vector (Clontech) or the same vector carrying the MLK2 cDNA along with P4-Luc and pCMV-β-galactosidase vectors. IFN-γ stimulation and reporter assays were carried out as in the above panels. **I, expression of the MLK2 protein in the transfectants.** Cell lysates were probed with GFP-specific antibodies after Western blotting. Wt, wild type; DN, dominant negative; WB, Western blot.

**Fig. 3.** CEP inhibits C/EBP-β-dependent gene expression through GATE. A, cebp−/− MEFs were transfected with the indicated plasmids (100 ng) along with P4-Luc (200 ng) and pCMV-β-galactosidase (50 ng) reporter. IFN-γ and CEP treatments were similar to those in other figures. These cell lysates were also used for Western blot analysis (B and C) and probed with C/EBP-β- and actin-specific antibodies. D, MLK3 promotes C/EBP-β-dependent gene expression in response to IFN-γ. RAW cells were transfected with expression vectors (100 ng) coding for HA-tagged wild type or catalytically inactive MLK3 proteins along with the P4-Luc reporter (200 ng). Luciferase activity was determined after various treatments. **E, expression of the MLK3 proteins was monitored using HA tag-specific antibodies. F, effect of MLK3 siRNAs on MLK3 expression.** Cell lysates were prepared after 36 h of transfection of the pSuppressor-Neo (pSup-Neo, empty expression vector) or the same expression vector coding for mouse (mMLK3 siRNA) and human (hMLK3 siRNA) siRNAs specific for MLK3. A comparable amount of the total protein (100 μg) was Western blotted with MLK3- and actin-specific antibodies. G, effect of MLK3 siRNAs on the IFN-γ-induced expression of P4-Luc and pIRE-Luc. RAW cells were transfected with equal quantities (100 ng) of reporter gene and the siRNA expression vectors. The cells were also transfected with 50 ng of pCMV-β-galactosidase reporter for normalizing variations in transfection efficiency. Normalized luciferase activities (as relative light units (RLU)) are shown. **H, effect of MLK2 on the IFN-γ-induced expression of P4-Luc.** Cells were transfected with the pEGFP-N1 vector (Clontech) or the same vector carrying the MLK2 cDNA along with P4-Luc and pCMV-β-galactosidase vectors. IFN-γ stimulation and reporter assays were carried out as in the above panels. **I, expression of the MLK2 protein in the transfectants.** Cell lysates were probed with GFP-specific antibodies after Western blotting. Wt, wild type; DN, dominant negative; WB, Western blot.

**Fig. 4.** Activation of MLK3 by IFN-γ. A, equal amounts (100 μg) of total cellular extract prepared from IFN-γ-stimulated RAW cells were separated by 10% SDS-PAGE, and a Western blot analysis was performed using native and phospho-MLK3-specific antibodies. Numbers above the panel show the minutes after IFN-γ treatment. B, effect of CEP on the IFN-γ-induced activation of MLK3. Cells were stimulated with IFN-γ for 4 h in the presence and absence of CEP, and protein extracts were prepared. A comparable quantity of cell extract from each was used for Western blot analyses with phospho-MLK3 or native MLK3-specific antibodies. pT, Thr(P); pS, Ser(P).

Because our earlier studies identified an important role for the Thr189 residue (a target for ERK1/2-induced phosphorylation), located in the regulatory domain 2 of C/EBP-β, in IFN-γ-induced gene expression (20, 21), we next examined whether CEP affected the IFN-γ-induced phosphorylation of C/EBP-β at Thr189. An antibody that detects the phospho-Thr189 form of C/EBP-β was used as a tool for detecting changes in ligand-induced phosphorylation. The specificity of anti-Thr(P)189 antibody is shown in Fig. 5C. In this experiment protein lysates from cebp−/− cells transfected with wild type (188GTPS191) and a corresponding alanine-substituted mutant (188GAAA191) showed that ERK1/2 were activated equivalently by IFN-γ in the presence and absence of CEP (Fig. 5A). Analysis of total ERK2 levels revealed a comparable expression (Fig. 5B). Thus, CEP did not inhibit ERK activation by IFN-γ.

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of C/EBP-β and stimulated with IFN-γ were used for a Western blot analysis with anti-Thr(P)189. A significant increase in IFN-γ-induced phosphorylation at Thr189 was observed with the wild type but not with the mutant protein. This difference was not due to differential expression of the wild type and mutant proteins (Fig. 5D). In the next experiment we examined whether CEP affected phosphorylation at Thr189 in RAW cells (Fig. 5E). In these studies U0126, a known inhibitor of ERK1/2, was used as an additional control. As expected, IFN-γ-induced phosphorylation of Thr189, which was blocked by U0126. In contrast to U0126, CEP had no effect on this process. These inhibitors did not affect either the basal phosphorylation or the expression (Fig. 5F) of C/EBP-β. Thus, CEP appears to target another step involved in IFN-γ-induced CEBP-β activation.

IFN-γ Negatively Regulates the Phosphorylation through a Serine Residue at the N Terminus of C/EBP-β—To further gain insight into the domain of C/EBP-β affected by CEP, we examined the sequence of C/EBP-β for potential phosphorylation sites. Recent studies (22) identified a new phosphoacceptor site, Ser64, in the transactivation domain of C/EBP-β. This motif exhibits putative homology to a motif found in c-Jun, a sub-strate for JNK. Because MLK3 regulates JNKs and these enzymes phosphorylate the N terminus of the Jun protein (23), we focused on this motif of C/EBP-β. However, inhibition of JNK activity using biochemical inhibitors or dominant negative JNK isoforms had no effect on the IFN-γ-induced gene expression through C/EBP-β (data not shown).

An affinity-purified antibody (anti-Ser(P)64) that specifically detects the phosphorylated Ser64 of C/EBP-β was generated recently (22). This antibody was used in Western blot analyses for monitoring changes in IFN-γ-induced phosphorylation at the Ser64 residue of C/EBP-β. To demonstrate the specificity of the anti-Ser(P)64 antibody in detecting phosphorylated C/EBP-β, we transfected wild type C/EBP-β and the S64A mutant into cebpb−/− MEFs and subjected the cell lysates to Western blot analysis. The antibody specifically detected wild type C/EBP-β but not the S64A mutant (Fig. 6A, top panel) even though comparable expression of the proteins was noted as analyzed by an antibody against the native protein (Fig. 6A, bottom panel). As expected, no protein was detected by either of the antibodies in cells transfected with the empty expression vector.

We next sought to determine the effects of IFN-γ in regulating Ser64 phosphorylation. RAW cells were stimulated with IFN-γ, and lysates were analyzed by Western blotting with the anti-Ser64 antibody (Fig. 6B, top panel). These blots were also probed with the native C/EBP-β antibody to monitor total C/EBP-β protein levels (Fig. 6B, middle panel). Interestingly IFN-γ treatment caused a significant decrease (35–40%) in the steady-state level of phosphorylation (Fig. 6B, bottom panel). Although we can see the decrease in phosphorylation maximally by 6 h it began to occur as early as 1 h after IFN treatment (data not shown). Therefore, we chose the 6-h time point for the rest of this study. This time point also correlates well with the early onset of the transcription of irf9 gene and ERK1/2 activation (20). In the light of these observations, we next studied the effect of CEP on Ser64 phosphorylation. In these studies we used the p38 mitogen-activated protein kinase inhibitor SB202190 as a negative control. As shown in Fig. 6C, IFN-γ caused a decrease in the phosphorylation of Ser64 in the control and SB202190-treated cells, and this dephosphorylation effect was blocked by CEP.

To further demonstrate that dephosphorylation of Ser64 is affected by MLK3, we transfected the cebpb−/− MEFs with a wild type C/EBP-β construct along with expression vectors encoding wild type MLK3 or a catalytically inactive MLK3. An empty vector-transfected sample was used as a control.
Cell lysates were prepared and analyzed for Ser\(^{64}\) phosphorylation by Western blotting (Fig. 6D). In the absence of IFN-\(\gamma\) treatment neither wild type nor mutant MLK3 caused an appreciable decrease in steady-state Ser\(^{64}\) phosphorylation. However, IFN-\(\gamma\)-induced dephosphorylation of Ser\(^{64}\) in Ser\(^{64}\) was blocked by the catalytically inactive MLK3. These data suggest a potential relationship between MLKs and IFN-\(\gamma\) in Ser\(^{64}\) dephosphorylation.

**MEKK1-independent Decrease in Phosphorylation at Ser\(^{64}\)**

Because our earlier studies have implicated a critical role for MEKK1 in regulating IFN-\(\gamma\)-stimulated responses through GATE (21), we were interested in knowing whether the IFN-\(\gamma\)-induced dephosphorylation at Ser\(^{64}\) similarly required MEKK1. For this purpose, wild type and mekk1\(^{-/-}\) cells were treated with IFN-\(\gamma\) and C/EBP-\(\beta\) was immunoprecipitated and analyzed by Western blotting using Ser\(^{64}\)-specific and C/EBP-\(\beta\)-specific antibodies. IFN-\(\gamma\)-induced dephosphorylation of C/EBP-\(\beta\) occurred in both cell types (Fig. 7A), indicating that this effect is independent of MEKK1. Our earlier studies have also shown that MEKK1 is necessary for IFN-\(\gamma\)-dependent activation of ERK1/ERK2 kinases, which phosphorylate Thr\(^{189}\) of C/EBP-\(\beta\) (20, 21). Therefore, it was of interest to determine whether IFN-\(\gamma\)-induced dephosphorylation of Ser\(^{64}\) required an intact GTPS motif. To examine this question, cebp\(^{b/-}\) MEFs were transfected with wild type C/EBP-\(\beta\) and an alanine-substituted mutant (188GTPS191) to 188GAAA191), and the IFN-\(\gamma\)-induced dephosphorylation of Ser\(^{64}\) in these two proteins was compared. There was no significant difference in steady-state phosphorylation and IFN-\(\gamma\)-induced dephosphorylation of Ser\(^{64}\) between the wild type and mutant forms of C/EBP-\(\beta\) (Fig. 7B). Thus, IFN-\(\gamma\)-induced Ser\(^{64}\) dephosphorylation is not dependent on an intact GTPS motif.

**CEP Inhibits Transcription through Ser\(^{64}\) of C/EBP-\(\beta\)**

To determine whether the Ser\(^{64}\) residue is critical for IFN-\(\gamma\)-induced expression and CEP inhibition, we used a S64A mutant (22) and measured its influence on IFN-\(\gamma\)-induced transcription through GATE (Fig. 8A). This experiment was performed in cebp\(^{b/-}\) MEFs to avoid interference from the endogenous C/EBP-\(\beta\). Transfection of wild type C/EBP-\(\beta\) or the S64A mutant caused similar increases in basal expression of the reporter, although S64A was significantly better \((p > 0.01)\) than the wild type at inducing transcription from GATE. More importantly, whereas the IFN-\(\gamma\)-induced expression of the reporter was sensitive to CEP inhibition in the presence of wild type C/EBP-\(\beta\), the S64A mutant was relatively resistant. The minor inhibitory effect on the S64A mutant suggests that MLKs can affect another IFN-\(\gamma\)-induced regulatory step.

Similarly we also measured the effect of the S64A mutation on induction of endogenous irf9 gene expression using reverse transcription-PCR. cebp\(^{b/-}\) MEFs were transfected with pcDNA3.1 or the same vector carrying wild-type C/EBP-\(\beta\) or the S64A mutant. Cells were stimulated with IFN-\(\gamma\) overnight in the presence and absence of CEP. As shown in Fig. 8B, irf9 mRNA was readily induced by IFN-\(\gamma\) in the presence of C/EBP-\(\beta\), which was suppressed by CEP. In contrast, the IFN-\(\gamma\)-induced expression of irf9 mRNA conferred by the S64A mutant was insensitive to CEP. Consistent with the reporter data,
approach. In these experiments, CEBPβ/H9252C/EBP-
One consequence of inhibition of the MLK3 (by CEP) is an atten-
tions specific IgG was used for IP reactions (irf9
PCR (input control). As shown in Fig. 9
of the soluble chromatin (without subjecting to IP) was used for
with
vectors coding for C/EBP-β and HA-p300 (lanes 3–6). Lanes 1 and 2 show the control samples in which pcDNA3.0 (the parent vector for p300) was
transfected along with C/EBP-β. After allowing the cells to recover
overnight, cells were stimulated with IFN-γ in the presence and ab-
sence of CEP. A comparable amount of cell extract from each sample
was subjected to Western blot (WB) and IP analyses with the indicated antibodies.

also analyzed by Western blotting for monitoring the expres-
sion of C/EBP-β and HA-p300 proteins. In the controls, an empty pcDNA3.0 vector (negative control for p300) was co-
transfected with a C/EBP-β expression vector. Following stimulation of cells with IFN-γ, cellular extracts were immu-
noprecipitated with HA tag-specific antibodies. No C/EBP-β
co-immunoprecipitated with these antibodies in the absence of HA-p300 protein, although copious amounts of C/EBP-β
were expressed in the lysates (Fig. 9B, lanes 1 and 2). In the
experimental samples HA-p300 and C/EBP-β proteins expressed
to a comparable extent (bottom panel, lanes 3–6). IFN-γ treatment readily stimulated the association of HA
p300 with C/EBP-β, which was strongly inhibited in the
presence of CEP (compare lanes 4 and 6). No significant effect of CEP on the basal interactions of HA-p300 and C/EBP-β
was observed under these conditions (lanes 3 and 5). Together
with the chromatin immunoprecipitation data, these observa-
tions show that CEP interferes with the IFN-γ-induced
association of transcriptional co-activators with C/EBP-β,
leading to the inhibition of GATE-driven transcription.

DISCUSSION

Among members of the C/EBP family, C/EBP-β has a unique
ability to interact with disparate transcription factors such as
NF-κB, pRb, STAT3, and STAT5 and regulate a number of
diverse physiologic processes (33). It is clear that signal-
directed post-translational modifications of C/EBP-β play a major
role in regulating its activity, C/EBP-β is phosphorylated at
various serine/threonine residues, each regulated by a specific
kinase (21, 34–38). More recently, a study has shown that
interleukin-3-dependent deacetylation of a lysine residue in the
basic region of C/EBP-β in a hematopoietic cell line contributes
to the induction of gene expression (39). In contrast to the
known positive regulatory effect of acetylation on other trans-
scription factors (40), this study showed that acetylation acts as
negative signal. Similarly sumoylation of C/EBP-β has been reported to inhibit the negative effects on transactivation (41). These and other studies (35, 37) indicate that the functional diversity of C/EBP-β is regulated in part by specific post-translational modifications in response to disparate extracellular stimuli. We have shown earlier that some members of the MAPK family, such as ERK1/2, are critical for regulating IFN-γ-stimulated GATE-driven gene expression (20), and they exert such an effect via a specific MAPK consensus motif located at Thr<sup>189</sup> of C/EBP-β. Surprisingly activation of this response was dependent on MEKK1, a regulator of stress-responsive MAPKs (21).

In an extension of our studies to understand the role of MAPKs in IFN-γ-induced transcriptional responses, we found that MLKs contribute to C/EBP-β-driven responses through GATE. MLKs are a family of structurally similar dual specificity kinases. Although grouped together on the basis of a leucine zipper, a signature motif, it is suspected that these kinases are distinctly regulated (23). In addition, the MLKs are expressed in a tissue-dependent manner. Their role in the regulation of neurodegeneration has been indicated by the observation that K252a, a metabolite found in the spent broths of bacterium Narcodiopsis sp., can inhibit these kinases and prevent experimentally induced neurodegeneration in animal models (42).

Using a semisynthetic inhibitor, CEP-11004 (an analogue of K252a), and a catalytically inactive MLK3 mutant (Fig. 3D) as well as gene-specific siRNAs (Fig. 3G) we showed that MLKs play an important role in IFN-γ-driven transcriptional responses. This effect is mediated through C/EBP-β, a factor known to bind to GATE (Fig. 1). MLKs appear to be dispensable for activating STAT1 (elicited by IFN-γ) and activator protein 1 (stimulated by epidermal growth factor)-driven responses, thus indicating their specificity in gene regulation. CEP did not inhibit ERK1/2, which is required for GATE-dependent C/EBP-β-driven responses (21), because a normal IFN-γ-induced activation of ERKs was observed in the presence of CEP (Fig. 5A). More importantly, CEP did not block the IFN-γ-induced phosphorylation at Thr<sup>189</sup> (Fig. 5). Previous studies have shown that MLKs act as upstream regulators of JNK and p38 MAPKs (23). However, the IFN-γ-driven response through GATE is refractory to inhibition by SB202190 (a pan-p38 kinase inhibitor), SP10006 (a pan-JNK inhibitor), and catalytically inactive JNK1/2 (data not shown). Similarly JNK and p38 activation was not observed in Western blot analyses using phospho-JNK- and phospho-p38-specific antibodies and extracts from IFN-γ-stimulated RAW cells (data not shown). These observations rule out the possibility that the MLK-regulated kinases JNK and p38 control C/EBP-β-driven responses through GATE. Lastly we have also shown that IFN-γ activates MLK3 (Fig. 4). Although it will be interesting to examine whether other MLKs are also activated by IFN-γ, antibodies specific for the native and phosphorylated forms of murine MLK1 and -2 are presently unavailable. MLK2, the other member of this group, is expressed only in skeletal muscle, brain, and testis, and co-expression of MLK2 did not significantly stimulate IFN-γ-induced expression of the irf9 promoter (Fig. 3H). Unlike the other family members, MLK3 is expressed ubiquitously. Because the irf9 gene is also induced in an IFN-γ-dependent manner in most tissues, our studies suggest that MLK3 is the major MLK species regulating IFN-induced gene expression. Furthermore experiments using siRNAs and a catalytically inactive MLK3 showed an important role for MLK3 in regulating GATE-driven transcription (Fig. 3G).

Although MLK3 is known to activate p38 MAPK and JNK in other cell types, the ultimate target transcription factors and the genes that respond to such signals have yet to be identified. In this study we identified a potential role for ligand (IFN-γ)-regulated MLK activation in activating a transcription factor (C/EBP-β). Surprisingly we found that MLK3 promotes a decrease in phosphorylation of C/EBP-β instead of its stimulation. This conclusion is supported by observations that both an enzymatically inert MLK3 and CEP (an MLK inhibitor) blocked dephosphorylation of the Ser<sup>64</sup> residue. More importantly, overexpressed MLK3 neither induced Ser<sup>64</sup> dephosphorylation nor activated transcription in the absence of IFN-γ stimulation. This observation suggests the requirement for a ligand-induced dephosphorylation activity to stimulate GATE-driven transcription. Indeed CEP interfered with the IFN-γ-induced recruitment of the transcriptional co-activator p300 to the irf9 promoter and physical association between the proteins (Fig. 9). Together these results suggest that IFN-induced GATE-driven transcription is dependent on MLK3 activity, which promotes a decrease in Ser<sup>64</sup> phosphorylation on C/EBP-β. Nevertheless our current understanding of the MLK3 effects is limited to the Ser<sup>64</sup> residue, and it is theoretically possible that additional sites on C/EBP-β are subject to regulation by MLK3 signals.

Transcriptional activation through dephosphorylation has been suggested in other studies. For example, dephosphorylation of the transcription factor nuclear factor in activated T cells by calcineurin (43–45) and homeodomain transcription factor Arix (46) has been shown to promote their transcription activating function. In contrast, dephosphorylation of Rb is linked to transcriptional repression. The nature of the MLK3-driven dephosphorylation activity is unclear at present. Although we suggest a role for phosphatase in this process, these data can also be explained by MLK3-driven deactivation of the kinase that constitutively phosphorylates Ser<sup>64</sup>. One likely candidate is Cdk2, which phosphorylates C/EBP-β in vitro (22). Indeed IFN-γ treatment has been shown to cause a decrease in Cdk2 activity (47). However, a Cdk2 dominant negative inhibitor did not exhibit a stimulatory effect on IFN-γ-induced GATE-driven transcription in a preliminary study (data not shown). One study showed that inhibition of protein phosphatase 2A was required for the IFN-γ-induced expression of the C1 inhibitor gene (48). However, we found that the protein phosphatase 2A inhibitors okadaic acid and cantharidin were highly toxic to cells even at low doses (2 μM), and in the presence of IFN-γ these cytotoxic effects were further augmented. Thus, we were unable to define the phosphatase activity responsible for C/EBP-β dephosphorylation. Thus, further studies are clearly required to identify the relevant activity in this response.

In keratinocytes and NIH 3T3 cells, C/EBP-β cooperates with Ras and promotes cellular transformation (18, 22). Indeed, co-expression of a constitutively active Ras and Raf stimulated gene expression through a consensus C/EBP-β binding site. Interestingly, the S<sup>64</sup>A mutant fails to respond to ras in a cellular transformation assay (22). Recent studies showed that MLK3 is required for cell proliferation in a B cell model. It seems to respond to Ras and Raf-induced signals in this scenario (31). Thus, like C/EBP-β, MLK3 also appears to be responding to both growth promoters and growth inhibitors. However, the downstream effects may be very different. In contrast to these results, GATE-dependent IFN-induced transcription occurs normally in the presence of inactive ras andraf mutants and in c-raf<sup>−/−</sup> cells (21). At this stage we are unclear about the upstream regulators of MLK3 in response to IFN-γ. We are currently investigating the upstream mechanisms involved in MLK3 activation. It seems to occur independently of c-Raf and MEKK1 because GATE-driven transcription occurs normally in the c-raf<sup>−/−</sup> cells, and IFN-γ-induced Ser<sup>64</sup> dephosphorylation is not inhibited in mekk1<sup>−/−</sup> cells. These observa-
tions suggest that IFN-γ induces MLK3 in a unique manner to regulate C/EBP-β-driven transcription. It will be interesting to examine what IFN-induced upstream signals impinge on MLK3.

MLK3 activation occurs maximally by 4 h and decreases by 6 h, but the decrease in phosphorylation at Ser⁶⁴ occurs at 6 h. This kinetic difference can be explained as follows. Activation of MLK3 probably leads to the delayed activation of downstream phosphatases, which then act on phosphorylated Ser⁶⁴. This suggestion is based on the fact that most known MAPK pathways involve signaling cascades that are dependent on intermediate transducers (49). At this stage we do not know what intermediate signaling factors are necessary to drive these pathways that culminate in the activation of a dephosphorylating activity. Furthermore activation of ERK1/2 by IFN-γ also follows similar late kinetics. Although the activation of ERK1/2 and MLK3 are not dependent on a common control point, they seem to occur coordinately. The convergence of these two signals on C/EBP-β and the recruitment of cofactors may then set the stage for activation of the irf9 gene. As mentioned earlier, the irf9 gene is induced by IFN-γ with delayed kinetics. The earliest onset of transcription is observed only 6–7 h after IFN-γ treatment. One explanation for this observation is the induction of sufficient levels of C/EBP-β in the cells by IFN-γ that must precede the initiation of irf9 transcription. Many cells (with the exception of liver, kidney, and adipose) express low levels of C/EBP-β that are elevated 4–6 h after IFN-γ treatment. This occurs both in vivo and in cell lines (12). Hence integration of MAPK-driven signals is kinetically coordinated with the elevation of C/EBP-β levels.

Interestingly IFN-γ induced partial dephosphorylation of Ser⁶⁴ (−35%) in several experiments. It appears that physiologically only a fraction of the total C/EBP-β participates in IFN-γ-induced responses, and the remaining fraction may participate in the regulation of other genes. Indeed only a small fraction of the total STAT protein is phosphorylated in response to the activating signal and is capable of exerting biological responses (5). Similarly only a small fraction of the cellular IFN-induced enzyme PKR (protein kinase activated by double-stranded RNA) is activated by phosphorylation to suppress protein translation effectively (50). A possible implication from these results is that signal-dependent phosphorylation (for growth promoters, such as Ras) and dephosphorylation (for growth inhibitors, such as IFN-γ) at Ser⁶⁴ may act as a regulatory switch for routing C/EBP-β into specific promoter complexes. Dephosphorylation of Ser⁶⁴ may thus permit the recruitment of specific transcriptional co-activators in an IFN-γ-dependent manner (Fig. 9). A postinduction rephosphorylation at Ser⁶⁴ following IFN-γ treatment may then “reset” the transcription switch in cells. Consistent with our interpretation, cell cycle-dependent dual phosphorylation of C/EBP-β at Ser⁶⁴ and Thr¹⁸⁹ by Cdc2 or Cdk2 controls the activation of C/EBP-β by Ras signaling (22). This result may also explain why IFN-γ circumvents Ras to activate ERK1/2 and therefore C/EBP-β (21); otherwise Ser⁶⁴ might remain constitutively phosphorylated and thus unable to activate GATE-dependent transcription. Importantly the IFN-γ-induced decrease in Ser⁶⁴ phosphorylation correlates kinetically well with the delayed expression of irf9 gene and the activation kinetics of ERK1/2 (20).

Finally the IFN-γ-induced decrease in the phosphorylation of Ser⁶⁴ seems to occur independently of the ERK consensus motif present in regulatory domain 2 of C/EBP-β. This conclusion is supported by three observations (Fig. 7). 1) In cells lacking mekk1, an upstream regulator of IFN-γ-induced ERK1/2 activities, a normal dephosphorylation of Ser⁶⁴ occurred in response to IFN-γ; 2) a C/EBP-β mutant lacking the ERK phosphorylation motif was dephosphorylated following IFN-γ treatment; and 3) CEP did not block the IFN-induced phosphorylation at Thr¹⁸⁹ of C/EBP-β. These observations also rule out a potential IFN-γ-induced cross-talk between MEKK1- and MLK3-driven signals. However, it is possible that these events can be coordinately regulated. That the TAD of C/EBP-β is a target for MLK3 is strongly supported by our observation that MLK3 can augment transcription driven by a chimeric transactivator bearing the wild type TAD of C/EBP-β but not S64A (Fig. 8). However, IFN-γ treatment did not augment the reporter gene expression driven by these chimeras (data not shown). Augmentation of GAL4-TAD-driven transcription by MLK3 without IFN-γ treatment, in contrast to the native C/EBP-β protein, also suggests the importance of ligand-inducible signals and the context of the transcription factor in driving specific effects. Based on the results of our previous study and the data presented here, we suggest that C/EBP-β is controlled by at least two independent IFN-γ-driven signaling pathways: one that promotes the phosphorylation at Thr¹⁸⁹ through an MEKK1-MEK1-ERK1/2 cascade and the other that decreases phosphorylation at the Ser⁶⁴ residue of the N terminus through MLK3, which may then activate a downstream dephosphorylating activity (Fig. 10). Although identification of this effector will be crucial for further defining the role in IFN-γ-induced responses through C/EBP-β, our studies provide the first evidence for the existence of a novel MLK-dependent pathway regulating C/EBP-β.

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A Role for Mixed Lineage Kinases in Regulating Transcription Factor CCAAT/Enhancer-binding Protein-β-dependent Gene Expression in Response to Interferon-γ

Sanjit K. Roy, Jon D. Shuman, Leonidas C. Platanias, Paul S. Shapiro, Sekhar P. M. Reddy, Peter F. Johnson and Dhananjaya V. Kalvakolanu

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