Signaling Pathways to the Assembly of an Interferon-β Enhanceosome

CHEMICAL GENETIC STUDIES WITH A SMALL MOLECULE* 

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Small molecules that modulate specific protein functions are valuable tools for dissecting complex signaling pathways. Here, we identified a small molecule that induces the assembly of the interferon-β (IFN-β) enhanceosome by stimulating all the enhancer-binding activator proteins: ATF2/c-JUN, IRF3, and p50/p65 of NF-κB. This compound stimulates mitogen-activated protein kinase kinase kinase 1 (MEKK1), which is a member of a family of proteins involved in stress-mediated signaling pathways. Consistent with this, MEKK1 activates IRF3 in addition to ATF2/c-JUN and NF-κB for the assembly of the IFN-β enhanceosome. MEKK1 activates IRF3 through the c-JUN amino-terminal kinase (JNK) pathway but not the p38 and IκB kinase (IKK) pathway. Taken together with previous observations, these results implicate that, for the assembly of an IFN-β enhanceosome, MEKK1 can induce IRF3 and ATF2/c-JUN through the JNK pathway, whereas it can induce NF-κB through the IKK pathway. Thus, specific MEKK family proteins may be able to integrate some of multiple signal transduction pathways leading to the specific activation of the IFN-β enhanceosome.

Most eukaryotic transcriptional enhancers contain binding sites for multiple distinct activator proteins. This enhancer organization is the basis of a combinatorial mechanism that is required for the selective activation of genes in response to extracellular signals or developmental cues (reviewed in Refs. 1–3). One model for studying the combinatorial gene regulation mechanism is provided by the enhancer of an IFN-β gene (reviewed in Ref. 4). The IFN-β enhancer includes an overlapping set of regulatory elements designated positive regulatory domains (PRDs)1 I through IV. PRDII, PRDIII, and PRDIV are recognized, respectively, by the transcription factors p50/p65 (NF-κB), IRF3, and ATF2/c-JUN. Based on the analyses of these activator proteins, it was proposed that they may form a stereospecific enhancer complex termed an enhanceosome.

Direct biochemical evidence has been obtained for this hypothesis (5–7). A functional interferon β (IFN-β) enhanceosome was assembled in vitro using the purified PRD-binding proteins. Synergistic activation of transcription by this enhanceosome requires the precise arrangement of activator binding sites in the enhancer DNA. Detailed mechanistic studies showed that transcriptional synergy is due to the cooperative assembly and increased stability of the enhanceosome. Furthermore, stereospecific assembly of the enhanceosome is critical for the efficient and specific recruitment of the transcriptional apparatus to the promoter and the formation of a stable preinitiation complex for synergistic activation.

These results demonstrate the importance of enhanceosome structure in transcriptional synergy, and they reveal new insights into the detailed mechanisms involved in this process with complex and intact enhancers. The next important questions include the nature of the signal transduction pathways leading to the activation of the enhanceosome. Little is known about the upstream kinases that coordinate the activation of the multiple transcription factors in the enhanceosome.

We have taken a chemical genetic approach that uses bioactive chemicals as molecular probes to study these signaling pathways (reviewed in Refs. 8–10). It is increasingly clear that, like genetic mutations, chemical ligands can either inactivate (e.g. trypoxin binding to histon deacetylase) or activate the protein (e.g. steroids binding to nuclear receptors). The first step in the chemical genetic approach is to screen for small molecules that affect specific cellular pathways. These chemicals can be used to elucidate the pathways by identification of the target proteins for which activities are affected.

In the present studies, we identified a small molecule that induces the IFN-β enhancer through the assembly of an enhanceosome. Based on the activation of mitogen-activated protein kinase kinase kinase 1 (MEKK1) by this compound, we showed that specific MEKK family proteins facilitate the assembly of the IFN-β enhanceosome: they can induce IRF3 and ATF2/c-JUN through the c-JUN amino-terminal kinase (JNK) pathway, and NF-κB through the IκB kinase (IKK) pathway.

MATERIALS AND METHODS

Cell Culture and Transfection—HeLa cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 2 mM l-glutamine, 100 μg/ml penicillin, and 100 μg/ml streptomycin. Cells were transfected with Lipofectamine reagent (Life Technologies, Inc.) using the plasmids indicated (see Figs 1–6).

Plasmids—GAL4-IRF3 and GFP-IRF3 were constructed by cloning IRF3 into pSG424 (11) and pEGFP-N1 (CLONTech), respectively. The G5E1b-CAT reporter plasmid containing five copies of GAL4 DNA binding sites with E1b TATA was described previously (12). Expression plasmids for MEKK1, MEKK3, MEKK4, MLK3, IκK, and p38 were described previously (13). The (PRDII)2-CAT, (PRDII–III)3-CAT, and

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1 The abbreviations used are: PRD, positive regulatory domain; IFN-β, interferon β; MEKK1, mitogen-activated protein kinase kinase kinase 1; JNK, c-JUN amino-terminal kinase; IKK, IκB kinase; bp, base pair(s); GFP, green fluorescence protein; WT, wild-type; HA, hemagglutinin; MLK, mixed lineage kinase.

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Assembly of the IFN-β Enhanceosome

A Small Molecule Induces the Assembly of the IFN-β Enhanceosome—To gain insight into the signaling pathways that induce the enhanceosome, we screened small molecules for their abilities to activate the intact IFN-β enhancer. HeLa cells were stably transfected with green fluorescence protein (GFP) as a reporter gene linked to the intact IFN-β enhancer. This cell-based assay system was used to screen the chemical inducers of the IFN-β enhancer. Among the 400 synthetic chemicals (Chembridge), high levels of fluorescent signal were detected in several, including CG-18 (Fig. 1A). CG-18 was chosen for further experiments because of its high level of induction.

The IFN-β enhancer was induced with increasing amounts of CG-18 in a dose-dependent manner, when CAT was used as a reporter gene (Fig. 1B).

To understand how CG-18 activates the IFN-β enhancer, we analyzed the effects of CG-18 on the activation of each PRD. The IFN-β enhancer contains multiple PRDs that bind distinct transcription factors, including NF-κB (PRDII), IRF3 (PRDIII–PRDIV), and ATF2/c-JUN (PRDIV) (4). HeLa cells were transfected with reporters containing multiple copies of each individual PRD (PRDII, lane 4; PRDIII, lane 6; PRDIV, lane 8).

Thus, the IFN-β enhancer is activated in response to CG-18 through induction of all the PRDs.

To further investigate the role of each PRD-binding transcription factor in the CG-18 induction of the IFN-β enhancer, HeLa cells were cotransfected with reporters linked to the IFN-β enhancer and plasmids expressing NF-κB, IRF3, and ATF2/c-JUN (Fig. 2B). Transfection of each PRD-binding factor slightly increased reporter gene expression from the IFN-β enhancer (compare lane 1 with lanes 3, 5, or 7). Under these conditions, treatment with CG-18 further potentiated these activator-dependent reporter gene activities (lanes 4, 6, and 8).

These results suggest that NF-κB, IRF3, and ATF2/c-JUN can be involved in the CG-18 induction of the IFN-β enhancer.

Activation of all the enhancer-binding proteins is not sufficient, and assembly of the enhanceosome is required for the specific activation of the IFN-β enhancer (4–7).
involvement of the enhanceosome in CG-18 induction of the IFN-β enhancer, we tested IFN-β enhancers in which a half or full helical turn of DNA was inserted between PRDI and PRDII (Fig. 2C). HeLa cells were cotransfected with 1 μg of (−110IFN-β)-CAT reporter plasmids. CAT activities were measured after treatment with CG-18 (10 μg/ml). B, HeLa cells were cotransfected with 1 μg of (−110IFN-β)-CAT reporter plasmid and 0.5 μg of an expression plasmid containing no insert (lanes 1 and 2), p50/p65 of NF-κB (lanes 3 and 4), IRF3 (lanes 5 and 6), or ATF2/c-JUN (lanes 7 and 8). CAT activities were measured after treatment with CG-18 (10 μg/ml). C, HeLa cells were transfected with 1 μg of a (−110IFN-β)-CAT plasmid containing an IFN-β enhancer with a wild-type sequence (WT), a 6-bp half helical insertion (I/II6), or a 10-bp full helical insertion (II/III10) between PRDI and II. CAT activities were measured after treatment with CG-18 (10 μg/ml).

![Figure 2](http://www.jbc.org/)

**FIG. 2.** CG-18 induces the assembly of the IFN-β enhanceosome. A, HeLa cells were transfected with 1 μg of (−110IFN-β), (PRDI-II), (PRDI–III), and (PRDIV)-CAT reporter plasmids. CAT activities were measured after treatment with CG-18 (10 μg/ml). B, HeLa cells were cotransfected with 1 μg of (−110IFN-β)-CAT reporter plasmid and 0.5 μg of an expression plasmid containing no insert (lanes 1 and 2), p50/p65 of NF-κB (lanes 3 and 4), IRF3 (lanes 5 and 6), or ATF2/c-JUN (lanes 7 and 8). CAT activities were measured after treatment with CG-18 (10 μg/ml). C, HeLa cells were transfected with 1 μg of a (−110IFN-β)-CAT plasmid containing an IFN-β enhancer with a wild-type sequence (WT), a 6-bp half helical insertion (I/II6), or a 10-bp full helical insertion (II/III10) between PRDI and II. CAT activities were measured after treatment with CG-18 (10 μg/ml).

A Small Molecule Is Capable of Stimulating the Kinase Activity of MEKK1—Treatment with CG-18 induced the assembly of the IFN-β enhanceosome by stimulating all the enhancer-binding proteins: NF-κB, IRF3, and ATF2/c-JUN (Figs. 1 and 2). Interestingly, CG-18 also stimulated transcriptional activity of p53 (data not shown) and a number of stress inducers (e.g. genotoxic agents) were shown to activate NF-κB (17–20), IRF3 (21), ATF2/c-JUN (22–26), and p53 (reviewed in Refs. 27–30). In response to environmental stresses, members of the MEKK protein family play a role in the induction of specific gene regulation pathways. For example, MEKK1 was demonstrated to activate ATF2/c-JUN (22–24), NF-κB (17, 18), and p53 (31). Thus, one possibility emerging from these observations is that CG-18 may activate all the IFN-β enhancer-binding proteins by stimulating the MEKK family member(s).

To test this possibility, the kinase activity of MEKK1 was measured after treatment with CG-18. MEKK1 and MLK3 were immunoprecipitated from cell lysates with antibodies raised against their specific sequences (16). Treatment with CG-18 increased both MEKK1 phosphorylation of JNKK1 and
autophosphorylation of MEKK1 (Fig. 3B; data not shown). Under these conditions, CG-18 failed to induce another MEKK family member, MLK3, indicating the specificity in the CG-18-dependent activation of MEKK1 (see also Fig. 5C). We also determined whether the activation of MEKK1 is correlated to the activation of IRF3 using the reporter linked to multiple copies of PRDI–III. Concomitant with MEKK1 activation, we detected the activation of IRF3 (and IFN-β enhancer) with increasing amounts of CG-18 in a dose-dependent manner (Fig. 3A; see also Fig. 1B). Thus, MEKK1 can be the specific part of CG-18 signaling pathways for the activation of IFN-β enhancer-binding proteins, including IRF3.

**MEKK1 Activates IRF3, in Addition to ATF2/c-JUN and NF-κB—**Based on the activation of MEKK1 by CG-18, we next examined whether MEKK1, like CG-18, can induce IRF3 in addition to the other enhancer-binding proteins ATF2/c-JUN (22–24) and NF-κB (17, 18). To this end, the transcriptional activity of the GAL4-IRF3 fusion protein was analyzed with a reporter plasmid containing GAL4 binding sites in the absence or presence of the ectopic expression of the active form of MEKK1 (32, 33). In these experiments, GALA-IRF3 protein levels were normalized to compare the transcriptional activities (data not shown). Expression of GALA-IRF3 resulted in low levels of the reporter gene expression (Fig. 4A, lane 2). Under these conditions, transcriptional activity of GALA-IRF3 was strongly stimulated by increasing amounts of MEKK1 in a dose-dependent manner (lanes 3–5). Consistently, treatment with CG-18 also markedly potentiated the transcriptional activity of GALA-IRF3 (lane 6). Thus, the transcriptional activity of IRF3 can be stimulated by MEKK1 like that of ATF2/c-JUN and NF-κB.

To further address the MEKK1-dependent IRF3 activation, we investigated the subcellular localization of IRF3 in the presence of MEKK1 (21). IRF3 was linked to GFP, transfected into cells, and then examined for MEKK1-induced changes in its subcellular localization (Fig. 4B). In uninduced cells, GFP-IRF3 was localized almost exclusively to the cytoplasm. Under these conditions, nuclear localization was observed in cells

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**Fig. 4. MEKK1 activates IRF3.** A, HeLa cells were cotransfected with 2 μg of G5E1b-CAT reporter plasmid and 0.5 μg of an expression plasmid containing no insert (0.5 μg; lane 2) or GAL4-IRF3 (lanes 2–6) in the absence (lanes 1, 2, and 6) or presence (0.5 μg in lane 3; 1 μg in lane 4; 2 μg in lane 5) of an expression plasmid for MEKK1. Transfected HeLa cells were treated with CG-18 (10 μg/ml) (lane 6). CAT activities were determined in extracts of transfected cells. B, HeLa cells were cotransfected with 1 μg of an expression plasmid for GFP-IRF3 and 2 μg of the expression plasmids for wild-type MEKK1 and mutant MEKK1 (K432M; MEKK1*). Transfected cells were treated with CG-18 (10 μg/ml) as indicated. The subcellular localization was then observed with a fluorescence microscope. C, HeLa cells were cotransfected with 2 μg of a G5E1b-CAT reporter plasmid and the following expression plasmids: 0.5 μg of mock (lanes 1) and 0.5 μg of GALA-IRF3 (lanes 2–5) plus mutant MEKK1 (K432M) (MEKK1*; 1 μg in lane 3; 2 μg in lane 4; 4 μg in lane 5). CAT activities were measured in extracts of transfected cells after CG-18 treatment (10 μg/ml), as indicated. D, HeLa cells cotransfected with an expression plasmid for HA-tagged IRF3 along with that for the mutant form of MEKK1 (K432M) (MEKK1*; lane 3). Transfected cells were labeled with [32P]orthophosphate for 6 h upon treatment with CG-18 (10 μg/ml). HA-IRF3 was immunoprecipitated for detection of phosphorylated IRF3.
transfected with MEKK1 expression plasmids. As expected, treatment with CG-18 also induced the nuclear localization of GFP-IRF3. These results, together with data from Fig. 4A, indicate that MEKK1 can induce the transcriptional activity of IRF3, along with its nuclear translocation.

To confirm the role of MEKK1 in the IRF3 activation, we tested whether a catalytically inactive form of MEKK1 (K432M) could act as a dominant inhibitor of CG-18 induction of the IRF3. To this end, the transcriptional activity of the GAL4-IRF3 fusion protein was analyzed with a reporter plasmid containing GAL4 binding sites in the presence of the ectopic expression of the mutant form of MEKK1 (Fig. 4C). The mutant MEKK1 significantly blocked the CG-18 induction of GAL4-IRF3 in a dose-dependent manner (Fig. 4C, lanes 3-5). Consistent with this, ectopic expression of the MEKK1 mutant inhibited the CG-18-dependent nuclear translocation of IRF3 (CG-18 + MEKK1*; Fig. 4B).

We also examined whether a dominant negative mutant of MEKK1 inhibited the in vivo phosphorylation of IRF3 after treatment with CG-18. HeLa cells were cotransfected with a MEKK1 mutant along with HA-tagged IRF3. Transfected cells were labeled with radioactive orthophosphate upon CG-18 treatment, and IRF3 protein was immunoprecipitated with anti-HA antibody. As shown in Fig. 4D, phosphorylation of IRF3 was induced by CG-18 (lane 2). This induced phosphorylation of IRF3 was significantly attenuated with ectopic expression of an MEKK1 mutant (CG-18 + MEKK1*; lane 3). Taken altogether with data from Fig. 4, these results indicate that MEKK1 can activate IRF3, whereas its dominant mutant blocks the activation of IRF3 in response to CG-18.

MEKK1 and MEKK3, but not MEKK4 and MLK3, Induce the Assembly of the Enhanceosome in the IFN-β Enhancer—MEKK1, like CG-18, can stimulate all the enhanceosome components (IRF3, ATF2/c-JUN, and NF-κB). Thus, we examined whether MEKK1 could induce all the PRDs for the assembly of an IFN-β enhanceosome (Fig. 5A). HeLa cells were cotransfected by the reporters linked to multiple copies of PRDII, PRDII-III, or PRDIV along with the expression plasmid for the active form of MEKK1. MEKK1 activated reporters containing multiple copies of each PRD (PRDII, lane 2; PRDII-III, lane 4; PRDIV, lane 6). Under these conditions, intact IFN-β enhancer was also stimulated by MEKK1 (lane 8). Thus, MEKK1 can induce the IFN-β enhancer by activating all the PRD-binding transcription factors.

To assess the involvement of the enhanceosome in MEKK1 induction of the IFN-β enhancer, we tested IFN-β enhancers in which a half or full helical turn of DNA was inserted between PRDI and II (Fig. 5B). HeLa cells were cotransfected by CAT reporters containing the intact or phasing mutant IFN-β enhancers along with an expression plasmid for MEKK1. Intact IFN-β enhancer was activated by MEKK1 (lane 2). In contrast, the level of MEKK1 induction decreased with insertion of a half helical turn between PRDI and PRDII (lane 4). Insertion of a full helical turn restored the activity of the IFN-β enhancer (lane 6). Thus, the stereospecific alignment of each activated PRD-binding factor is required for the MEKK1-induced transcriptional activity of the IFN-β enhancer.

The MEKK family of enzymes shares a conserved carboxyl-terminal catalytic domain (32, 33). Thus, MEKK members other than MEKK1 may also activate the IFN-β enhancer. To address the specificity in the activation of an IFN-β enhancer, we examined several MEKK family members for their abilities to induce the IFN-β enhancer (Fig. 5C). HeLa cells were cotransfected with the CAT reporter linked to the intact IFN-β enhancer and expression plasmids for MEKK1, MEKK3, MEKK4, and MLK3. As expected, the IFN-β enhancer was stimulated by MEKK1 (lane 2). However, MEKK4 and MLK3 failed to stimulate reporter gene activity from the IFN-β enhancer (lanes 3 and 4). MEKK4 and MLK3 are functionally active, because their coexpression with JNK in parallel experiments resulted in the marked activation of JNK, which were determined in immunocomplex kinase assays using the substrate GST-c-JUN protein (data not shown). Under these conditions, the transcriptional activity of the IFN-β enhancer was strongly potentiated by MEKK3 (lane 5). This MEKK3 Induction was specifically observed with the IFN-β enhancers containing the correct phasing of transcription factor binding sites (data not shown). Thus, the failure of MEKK4 and MLK3 to induce the IFN-β enhancer indicated that there is specificity in the induction of an IFN-β enhancer by MEKK family proteins.
MEKK-JNK Pathway Is Involved in the Activation of IRF3 in Response to CG-18—Previous results suggest that MEKK1 and MEKK3 can activate ATF/c-Jun through the JNK pathway and NF-κB through the IKK pathway (13, 17, 18, 22–24). Thus, to understand the MEKK downstream signaling pathways to the IFN-β enhanceosome, we examined the possible role of JNK, IKK, and p38 in the activation of IRF3 (Fig. 6A). To this end, increasing amounts of JNK, IKKβ, and p38 expression plasmids were cotransfected with HeLa cells with a GAL4-IRF3 expression plasmid and a CAT reporter plasmid containing GAL4 sites. Treatment with CG-18 induced reporter gene expression by GAL4-IRF3 (lane 2). This induction further increased with the expression of JNK in a dose-dependent manner (lanes 3 and 4). In contrast, expression of IKKβ and p38 did not significantly potentiate transcriptional activity of IRF3 (lanes 5–8). Under these conditions, IRF3 was not activated by JNK in the absence of CG-18 (data not shown), consistent with previous results that JNK is inactive in the absence of inducing stimuli (25, 26).

Next, we directly determined the kinase activity of JNK and IKK after treatment with CG-18. HeLa cells were transfected with an expression plasmid for flag-tagged JNK, IKKβ, and p38. After treatment with CG-18, kinases were immunoprecipitated and their amounts were normalized by immunoblot analysis (data not shown). The kinase activity was then measured in vitro using purified recombinant his-tagged IRF3 or GST-IκBα protein as a substrate. CAT activities were measured in extracts of cells transfected with 2 μg of a (PRDI–III) CAT reporter plasmid and the following expression plasmids: 0.5 μg of mock (lane 1), 0.5 μg of IRF3 (lanes 2–7), and IRF3mt containing mutations in IRF3 phosphorylation sites (lanes 8 and 9) plus 0.5 μg of JNK (lanes 3, 6, and 9), or p38 (lanes 4 and 7) in the absence or presence of 0.5 μg of MEKK1 as indicated. D, HeLa cells were cotransfected with an expression plasmid for GFP-IRF3mt along with the expression plasmids for MEKK1 and JNK as indicated in C. The subcellular localization was then observed with a fluorescence microscope. E, HeLa cells transfected with an expression plasmid for HA-tagged IRF3 (wild-type in lanes 1 and 2, mutant in lanes 3 and 4) along with the expression plasmids for MEKK1 and JNK as indicated in C. Transfected cells were labeled with [32P]orthophosphate, and HA-IRF3 was immunoprecipitated for detection of phosphorylated IRF3.

Fig. 6. MEKK-JNK pathway is involved in the activation of IRF3. A, HeLa cells were cotransfected with 2 μg of G5E1b-CAT reporter plasmid and 0.5 μg of an expression plasmid containing no insert (0.5 μg; lane 1) or GAL4-IRF3 (lanes 2–8) in the absence (lane 2) or presence (1 μg in lanes 3, 5, and 7; 2 μg in lanes 4, 6, and 8) of an expression plasmid for JNK, IKKβ, or p38 as indicated. CAT activities were determined in extracts of transfected cells after treatment with CG-18. B, HeLa cells were transfected with an expression plasmid for flag-tagged JNK, IKKβ, or p38. After 6 h of treatment with CG-18 (10 μg/ml), kinases were immunoprecipitated and their amounts were normalized by immunoblot analysis (data not shown). The kinase activity was then measured in vitro using purified recombinant his-tagged IRF3 or GST-IκBα protein as a substrate. C, CAT activities were measured in extracts of cells transfected with 2 μg of an (PRDI–III) CAT reporter plasmid and the following expression plasmids: 0.5 μg of mock (lane 1), 0.5 μg of IRF3 (lanes 2–7), and IRF3mt containing mutations in IRF3 phosphorylation sites (lanes 8 and 9) plus 0.5 μg of JNK (lanes 3, 6, and 9), or p38 (lanes 4 and 7) in the absence or presence of 0.5 μg of MEKK1 as indicated. D, HeLa cells were cotransfected with an expression plasmid for GFP-IRF3mt along with the expression plasmids for MEKK1 and JNK as indicated in C. The subcellular localization was then observed with a fluorescence microscope. E, HeLa cells transfected with an expression plasmid for HA-tagged IRF3 (wild-type in lanes 1 and 2, mutant in lanes 3 and 4) along with the expression plasmids for MEKK1 and JNK as indicated in C. Transfected cells were labeled with [32P]orthophosphate, and HA-IRF3 was immunoprecipitated for detection of phosphorylated IRF3.
Assembly of the IFN-β Enhancosome

Fig. 7. A model for the signaling pathways leading to the activation of the IFN-β enhancer. The enhancosome can be specifically assembled through coordinated activation of distinct transcription factors (ATF2/c-JUN, IRF3, and NF-κB) for the induction of an IFN-β enhancer. IRF3 and ATF2/c-JUN can be activated through the JNK pathway, and NF-κB can be activated through the IKK pathway. The activation of IRF3 may require another inducible kinase that directly phosphorylates Ser and Thr clusters (see “Discussion” for details). MEKK1 and MEKK3 can coordinate the multiple downstream signaling pathways (e.g. JNK and IKK) for the induction of the IFN-β enhancosome.

along with MEKK1 failed to induce IRF3 (lane 7). In contrast, coexpression of JNK with MEKK1 (or MEKK3) synergistically stimulated the IRF3 transcriptional activity (lane 6; data not shown).

Several Ser and Thr residues were identified to be phosphorylated for the activation of IRF3 (34). Thus, we tested transcriptional activity (Fig. 6C), nuclear translocation (Fig. 6D), and in vivo phosphorylation (Fig. 6E) of an IRF3 mutant (S396A, S398A, S402A, T404A, S405A) in which phosphorylation sites at Ser and Thr were substituted by Ala. In contrast to wild-type IRF3, the mutated IRF3 protein (IRF3mut) was not efficiently activated by JNK and MEKK1 (Fig. 6C; compare lanes 6 and 9). Under these conditions, overexpression of MEKK1 and JNK did not significantly induce the nuclear localization of an IRF3 mutant (Fig. 6D; compare with wild-type IRF3 in Fig. 4B). Consistent with these results, phosphorylation of IRF3 was significantly attenuated with mutation of Ser and Thr residues (Fig. 6E; compare lanes 2 and 4). Thus, Ser and Thr clusters in IRF3 are important for its activation by the MEKK-JNK pathway.

DISCUSSION

Here, we have identified a small molecule, CG-18, that induces the assembly of the IFN-β enhancosome by stimulating each of the PRD-binding transcription factors: NF-κB (p50/p65), IRF3, and ATF2/c-JUN (Figs. 1 and 2). Although its exact action mechanisms remain to be determined, CG-18 is capable of stimulating MEKK1, which is a member of the MEKK protein family, thereby inducing downstream signaling pathways in response to a variety of environmental stresses (Fig. 3). There is specificity in the CG-18 activation of MEKK family members, because CG-18 failed to stimulate the kinase activity of MLK3 (Fig. 3). Based on this specific activation of MEKK1, our studies extended to the signal transduction pathways leading to the induction of an IFN-β enhancosome. MEKK1, like CG-18, can activate IRF3 (Figs. 4 and 6) in addition to ATF2/c-JUN (reviewed in Refs. 22–24) and NF-κB (reviewed in Refs. 17, 18) for the assembly of the enhancosome. Furthermore, we showed that JNK and IKK pathway can activate IRF3 (and ATF2/c-JUN; see Refs. 22–24) and NF-κB in response to CG-18, respectively (Fig. 6). Taken together, these results are consistent with the idea that specific MEKK family proteins (e.g. MEKK1 and MEKK3) can induce IRF3 and ATF2/c-JUN through the JNK pathway, and NF-κB through the IKK pathway, for the assembly of an IFN-β enhancosome (Fig. 7). Induced assembly of the enhancosome by a single protein, MEKK1 (or MEKK3), provides an intriguing explanation for how a specific signal can simultaneously activate distinct transcription factors through multiple signaling pathways under the context of the natural enhancer.

Most of these results were obtained with ectopic expression of kinase(s), but high levels of specificity seem to be involved in the activation of the IFN-β enhancer under our experimental conditions: 1) induction was observed by MEKK1 and MEKK3 with the IFN-β enhancers containing the correct phasing of activator binding sites (wild-type (WT) and I/I10), but not with the helical phasing mutant enhancer (I/I6) (Fig. 5B); 2) MEKK4 and MLK3, but not MEKK1 and MEKK3, failed to induce the IFN-β enhancer (Fig. 5C); 3) JNK, but not p38 and IKK, activated IRF3 (Fig. 6A); and 4) phosphorylation mutant IRF3 failed to support the activation responses by JNK and MEKK1 (Fig. 6, C–E). Moreover, some experiments were performed with limiting amounts of kinases (JNK, p38, and MEKK1), and the results showed highly synergistic activation of IRF3 by JNK and MEKK1 but not by p38 and MEKK1 (Fig. 6C). Importantly, treatment with CG-18 can induce MEKK1 but not MLK3 (Fig. 3), and it can induce phosphorylation of IRF3 by JNK but not by p38 (Fig. 6D). Thus, CG-18 is the inducer of specific signaling pathways, but it is not the inducer of an entire range of signaling pathways under our experimental conditions.

IRF3 is activated by a variety of DNA-damaging agents, including UV, doxorubicin, mitomycin C, etoposide, and cisplatin (21). JNK is also known to be activated in response to cellular stresses induced by various DNA-damaging agents. Furthermore, it was recently shown that viral infection, a potent inducer of IRF3, activates JNK and treatment with lipopolysaccharide, a potent inducer of JNK, can activate IRF3 (35–37). Consistently, we detected phosphorylation of IRF3 by JNK in response to viral infection (data not shown), although the exact role of JNK in the IRF3 activation during viral infection remains to be established. Thus, these observations suggest that various kinds of inducing agents may converge on the activation of a JNK-IRF3 pathway.

Interestingly, various DNA-damaging agents and CG-18 were found to activate p53 in addition to IRF3 (21; data not shown). Several kinase pathways, including MEKK-JNK, were shown to phosphorylate p53 (31; reviewed in Refs. 27–30), and multiple kinases have been proposed to control p53 activity. For example, ataxia telangiectasia mutated kinase phosphorylates p53 following ionizing radiation but not following UV irradiation, suggesting the involvement of other kinases such as ataxia telangiectasia mutated kinase-related kinase and JNK in the UV induction of p53. Furthermore, p53 can be phosphorylated by multiple kinases in the course of activation; for example, different kinases are involved in the early (e.g. ataxia telangiectasia mutated kinase) and later (e.g. ataxia telangiectasia mutated kinase-related kinase) phases of p53 phosphorylation in response to γ-radiation. Related to these observations, Ser and Thr clusters that are critical for the activation of IRF3 by the MEKK-JNK pathway do not exactly match with the proposed phosphorylation sequences for JNK. Thus, similar to the situation for p53, it is plausible that JNK phosphorylates IRF3 at certain residues, and this event may facilitate the recruitment of a kinase that directly phosphorylates Ser and Thr clusters for the activation of IRF3. This idea is supported by the results that activation of the MEKK-JNK pathway strongly augmented the phosphorylation of IRF3, and, under these conditions, mutation of Ser and Thr clusters inhibited, but not completely, MEKK/JNK-dependent phosphorylation of IRF3 (Fig. 6E; data not shown). Thus, the MEKK-JNK pathway seems to phosphorylate IRF3 at distinct residues and, indeed, viral infection induces phosphorylation at a num-
A key issue in understanding inducible gene regulation is to elucidate how signaling specificity is achieved when many of the same core signaling pathways are activated by stimuli that induce different transcription factors. Signaling specificity can result from a unique combination of signaling pathways, because gene responses may reflect an integration of outputs from all the pathways activated by a single inducing agent. Thus, the expression of a gene may depend on the simultaneous interaction of a specific combination of induced transcription factors with the control DNA elements (reviewed in Refs. 1–4). Furthermore, it is possible that induction of a given transcription factor is tightly controlled by a specific combination of signaling pathways. Related to this, p53 and IRF3 are inducible by various DNA-damaging agents and CG-18, and by JNK with overexpression of MEKK1, but these transcription factors can not be significantly activated by some other JNK-inducing agents (e.g., tumor necrosis factor-α). These observations can be explained by a model that multiple kinases are involved in the specific and optimal activation of p53 and IRF3 in cells. These multiple regulations may provide a self-editing mechanism for ensuring that specific transcription factors are activated while a variety of signaling pathways are induced. In addition, the cooperativity of these multiple regulations could determine the threshold levels required for the activation of a transcription factor, and these levels could be inducer-specific.

Another emerging idea for signaling specificity is the formation of highly specific signaling complexes (reviewed in Refs. 39, 40). In the combinatorial mechanism, formation of specific signaling complexes could provide the basis for cross-talk in multiple signaling pathways induced by stimuli. These complexes may result from the assembly of signaling molecules on anchor or scaffold proteins that localize their binding partners to specific subcellular compartments or to specific substrates. Thus, one possible way to provide high levels of specificity in the enhancer function is the induction of specific enhancer complex (enhancosome) by a specific signaling complex (signalosome). Involvement of scaffold proteins in such enhancer complexes (HMG [IY] and LEF-1) and signaling complexes (Ste5, Pbs2, JIP1, and MP1) may provide the potential to achieve a high degree of regulatory flexibility with limited numbers of regulators (reviewed in Refs. 1–4, 39, 40). It is tempting to predict that scaffolding/adaptor proteins could be involved in the formation of specific signaling complexes that activate the distinct transcription factors in the IFN-β enhancosome and that the relative potencies of various agents to induce the IFN-β enhancer could be due to their abilities to induce the specific signaling and enhancer complexes. Our studies using a chemical genetic approach have begun to provide a framework for understanding how IFN-β enhancer can be activated by specific signaling pathways.

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