Interaction between c-Rel and the Mitogen-activated Protein Kinase Kinase Kinase 1 Signaling Cascade in Mediating \( \kappa B \) Enhancer Activation

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The Rel family of transcription factors are important mediators of various cytokine stimuli such as interleukin (IL)-1, tumor necrosis factor (TNF)-\( \alpha \), and CD28 costimulation in T cell effector responses. These stimuli induce Rel family DNA-binding activity to the \( \kappa B \) enhancer and CD28 response elements of many cytokine gene promoters leading to cytokine production. Consistent with the importance of Rel family induction during immune responses, c-Rel knockout mice exhibit profound defects in T cell functions including IL-2 secretion and T cell proliferative responses to CD28 plus T cell receptor (TCR) costimulation. The nuclear protein kinase, c-Jun NH2-terminal kinases (JNKs)/stress-activated protein kinases, are also activated by TNF-\( \alpha \), IL-1, and CD28 costimulation. Because of the common regulation of c-Rel and JNK1 by these agents in T cells, we investigated the role of JNK1 in c-Rel activation. We found that MAP kinase kinase kinase (MEKK) 1, a JNK1 activator, induced transcription from the human immunodeficiency virus-1 long terminal repeat and IL-2R\( \alpha \) promoters in a \( \kappa B \)-dependent manner. Coexpression of \( \kappa B \)\( \alpha \), a c-Rel inhibitor, inhibited the MEKK1-induced transcriptional activity. JNK1 synergized with MEKK1 in activating transcription from a \( \kappa B \)-driven heterologous promoter. Furthermore, JNK1 associated with c-Rel in vivo in Jurkat T cells by immunoprecipitation assays and bound directly to c-Rel in a yeast two-hybrid assay. c-Rel also competed with c-Jun in vitro kinase assays. However, JNK1 did not phosphorylate c-Rel, NF-\( \kappa B \), and I\( \kappa B \)\( \alpha \) in vitro, indicating that c-Rel may serve as a docking molecule to allow JNK1 phosphorylation of certain Rel-associated proteins. Transactivation of the IL-2R\( \alpha \) and HIV-\( \kappa B \)-driven promoters by c-Rel was augmented by coexpression of MEKK1. These results demonstrate the first significant role for the MEKK1 kinase cascade module in c-Rel-mediated transcription.

Characterization of signal transduction pathways mediating cellular stress responses has expanded rapidly with the discovery of multiple, parallel, mammalian mitogen-activated protein kinase (MAPK)\(^1\) modules homologous to those in yeast (reviewed in Refs. 1–4). Recently, a growing family of mammalian kinases, c-Jun NH2-terminal kinases (JNKs) (5, 6), and their rat homologs, stress-activated protein kinases (7), were identified based upon their activation by environmental stimuli such as UV light and the protein synthesis inhibitor anisomycin. Subsequently, kinase activation by osmotic shock (6), CD28 plus T cell receptor (TCR) costimulation (8), proinflammatory cytokines such as TNF-\( \alpha \) (9, 10) and IL-1 (11), and DNA-damaging agents (12) has been demonstrated. Delineation of the pathway linking receptors to JNK activation reveals similarity to the Raf \( \rightarrow \) MEK \( \rightarrow \) MAPK pathway. MEKK1 activates JNK/MEKK4/SEK1, a MEK homolog, which then activates JNK1 (13–15). MEKK1 activation is apparently specific for the JNK cascade at physiological levels as only overexpression of MEKK1 induces MAPK activity (14–16).

While many of the activating inputs of JNK have been defined, few substrate outputs have been identified. JNK1 has been shown to bind, phosphorylate, and activate the transcription factors c-Jun (5), ATF2 (12, 17, 18), and Elk-1 (19). Many of the agents which activate JNK1 such as TNF-\( \alpha \), IL-1, UV light, and CD28 plus TCR costimulation also activate the Rel/\( \kappa B \)-\( \kappa B \) family of transcription factors causing transcriptional activation of promoters containing \( \kappa B \) enhancers such as the HIV-1 LTR and IL-2R\( \alpha \) promoters. Because of the common regulation of JNK1 and c-Rel by a number of agents, we investigated the role of the MEKK1/JNK1 kinase module in \( \kappa B \) enhancer activation. In this report, we demonstrate the induction of the HIV-1 LTR and IL-2R\( \alpha \) promoter by the MEKK1 signaling cascade. Furthermore, JNK1 directly bound c-Rel, and MEKK1 synergized with c-Rel to activate transcription from \( \kappa B \)-driven promoters.

MATERIALS AND METHODS
Cells, Transfections, and Antibodies—Jurkat cell culture was performed as described (20). 293T cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and 1 \( \times \) penicillin/streptomycin. 293T cells were plated at a density of 2 \( \times \) 10\(^6\) cells/100-mm dish and transfected the next day using the calcium phosphate precipitation protocol (Specialty Media, Inc.). CAT assays were done as described previously (21, 22). Anti-c-Rel antibodies Ab1135 and Ab265 were generous gifts of N. Rice (NCI-FCRDC). The COOH-terminal anti-ERK-2 antibody and anti-JNK antibodies \( \alpha -1 \), \( \alpha -4 \), and \( \alpha -5 \), which recognize the JNK1 COOH terminus, JNK1 NH2-terminus. Reprints and correspondence should be addressed to: Dept. of Microbiology and Immunology, M929, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030. Tel.: 713-798-4665; Fax: 713-798-3700. §A Scholar of the Leukemia Society of America.

\(^{1}\)The abbreviations used are: MAPK, mitogen-activated protein kinase; JNK, c-Jun NH2-terminal kinase; MEKK, MAP kinase kinase kinase; GST, glutathione S-transferase; TCR, T cell receptor; TNF, tumor necrosis factor; IL, interleukin; IL-2R, interleukin-2 receptor; HIV, human immunodeficiency virus; LTR, long chain repeat; RSV, Rous sarcoma virus; CMV, cytomegalovirus; CAT, chloramphenicol acetyl transferase; MOPS, 4-morpholinepropanesulfonic acid; DTT, dithiothreitol; X-gal, 5-bromo-4 chloro-3-indolyl \( \beta \)-D-galactoside; MEK, mitogen-activated extracellular response kinase kinase.
terminus, and JNK1 recombinant protein, respectively, were purchased from Santa Cruz Biotechnology. Polyclonal rabbit anti-JNK1 antibodies, Ab101 and Ab102, were raised against a peptide containing the 18 carboxyl-terminal amino acids of human JNK1. Polyclonal rabbit anti-p38 antibody, Ab221, was raised against a peptide containing the 12 carboxyl-terminal amino acids of human p38.0.

**Two-cycle Immunoprecipitations—** Jurkat cells were incubated in methionine-free medium with 2% fetal bovine serum, 1% penicillin/streptomycin, 1% glutamine, and 1 μM β-mercaptoethanol for 45–90 min at 37°C and resuspended at 1–2.5 × 10^6 cells/ml in the same medium plus 100 μM [35S]methionine. Two-cycle immunoprecipitations were performed as described previously (20). Briefly, the cells were incubated in radiomimetic protein buffer for 30°C. The invasion fraction (of pH 8.0), 150 mM NaCl, 1% deoxocholate, 1% Nonidet P-40, 0.15% SDS, 5% glyceral, 1 mM EDTA, 10 mM K2HPO4, 500 μM phenylmethylsulfonyl fluoride, 3.3 μg of aprotinin, 10 μM leupeptin, 10 μM NaF, 1 mM Na3VO4 by repeatedly drawing through a 21.5-gauge needle and syringe. 40 μl of aprotinin, 0.5 mM phenylmethylsulfonyl fluoride, 3.3 μg of aprotinin, 5 μM leupeptin, 10 μM NaF, 1 mM Na3VO4 by repeatedly drawing through a 21.5-gauge needle and syringe. The extract was clarified by spinning at 14,000 rpm for 15 min. The precipitates were washed four times in 1 ml of lysis buffer, three times with 1 ml of Buffer A (20 mM MOPS, pH 7.0, 2 mM EGTA, 50 mM β-glycerophosphate, 1% Triton X-100, 10% glycerol, 1 mM DTT, 5 μM leupeptin, 3.3 μg/ml aprotinin, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM Na3VO4, 5 μM NaF, and 10 μM p-nitrophosphatase) on ice to remove the SDS was removed from the gel by washing with 200 ml of solution I (20% isopropanol, 50 mM Tris (pH 8.0)) for 1 h followed by washing with 200 ml of solution II (50 mM Tris (pH 8.0), 1 mM DTT) for 1 h at room temperature. The proteins were denatured by incubating the gel twice for 1 h in 80 ml of solution III (50 mM Tris (pH 8.0), 5 mM DTT, 6 μg guanidine hydrochloride) at room temperature and renatured in 200 ml of solution IV (0.045 Tween 20, 1 mM DTT, 50 mM Tris (pH 8.0)) at 4°C for 16–18 h with 5 buffer changes. The gel was first incubated in 15 ml of kinase buffer (20 mM HEPES (pH 7.6), 20 mM MgCl2, 20 mM β-glycerophosphate, 0.1 mM Na3VO4, 2 mM DTT, 20 mM p-nitrophosphatase) for 30–60 min at 4°C, then subjected to a kinase reaction using 15 ml of kinase buffer containing 150 μCi of (γ-32P)ATP and 50 μM ATP for 30 min at room temperature. The gel was washed extensively with 200 ml of solution V (5% w/v trichloroacetic acid and 1% sodium pyrophosphate) until the radioactivity was negligible (four to five changes).

**c-Rel Competition Assay—** GST or GST-c-Rel (2–587) was incubated with the JNK1 (isolated as described in the immunocomplex kinase assays) for 35 min at room temperature. Then, GST-c-Rel (1–79) was added, and the kinase reactions performed as described in the immunocomplex kinase assays.

**RESULTS**

**MEKK1 Induces Transcriptional Activation of xB-driven Promoters—** As many common agents activate the MEKK1/JNK1 kinase module and the NF-kB transcription factor family, we examined the ability of the MEKK1/JNK1 module to drive transcription of promoters containing xB sites. MEKK1 was used because it preferentially activates JNK1, and overexpression of JNK1 alone induces no kinase activity. Transient transfections of Jurkat cells with IL-2Rα-CAT and HIV-CAT reporter constructs were performed. Cotransfection of ΔMEKK1, an activated form of MEKK1, and IL-2Rα-CAT or the HIV-CAT reporter resulted in 5- and 8-fold induction of CAT activities, respectively (Figs. 1, A and B, lanes 1 and 2). To confirm the xB sites as ΔMEKK1-responsive elements, ΔMEKK1 was cotransfected with the xB deletion reporters, HIV(xB)-CAT or IL2Rα(xB)-CAT (Fig. 1, A and B, lanes 5 and 6). Deletion of the xB sites from the full-length promoters significantly reduced ΔMEKK1-mediated induction. The HINV(xB)-CAT construct was functional as it was Tat-responsive. These data demonstrate that the xB sites in the IL-2Rα promoter and the HIV-LTR are MEKK1-responsive elements. ReLNF-xB family members are retained in the cytoplasm by various inhibitory molecules including IxBα. ReLNF-xB induction involves the phosphorylation and degradation of IxBα, resulting in the release of ReLNF-xB and subsequent translocation to the nucleus. To demonstrate that the ΔMEKK1-induced transcriptional responses involve the Rel family of transcription factors, ΔMEKK1 and IxBα were co-transfected into Jurkat cells with the IL-2Rα-CAT and HIV-CAT reporter constructs. While ΔMEKK1 alone induced transcription from these reporters (Fig. 1), coexpression of IxBα inhibited ΔMEKK1-mediated induction (Fig. 1, A and B, lane 3). These results demonstrate that the NF-xB family of transcription factors are necessary for MEKK1-induced transcriptional effects from these xB-driven promoters.

**MEKK1, which has a growth factor-induced, Ras-dependent kinase activity (29) and a kinase domain that binds directly to Ras in vitro (30), was first identified as an upstream kinase in the MAPK cascade (28). However, recent work in several laboratories has positioned MEKK1 as an upstream kinase in the JNK cascade. Induction of stably transfected MEKK1 resulted in JNK and not MAPK activity. Only overexpression of MEKK1 induced some MAPK activity (14, 16, 31, 32) suggesting that MEKK1 induction of the JNK cascade is more physiologically relevant. To substantiate JNK1 involvement in the ΔMEKK1-induced NF-xB transcription which is shown in Fig. 1, suboptimal amounts of ΔMEKK1 alone, JNK1 alone, or ΔMEKK1 plus JNK1 were co-transfected with the 2xNF-xB-CAT reporter construct, 6tkCAT. JNK1 increased ΔMEKK1-induced trans-
transcription of these promoters is mediated by the Rel/NF-

kappa B Enhancer Activation

Fig. 1. MEKK1 induces kB enhancer-dependent IL-2Rα and HIV-LTR transcription. A, 5 µg of the IL2Rα-CAT or IL2Rα(ΔxB)-CAT (22) reporter was transfected into Jurkat cells with 5 µg of pEE-CMV (lanes 1 and 4), 5 µg of pCMV-ΔMEKK1 (lanes 2 and 5), or 5 µg of pCMV-ΔMEKK1 + 10 µg of pRSV-IxBα (lanes 3 and 6) (21) by electroporation. B, 3 µg of the HIV-CAT or HIV(ΔxB)-CAT (34) reporter was transfected into Jurkat cells with 5 µg of pEE-CMV (lanes 1 and 5), 5 µg of pCMV-ΔMEKK1 (lanes 2 and 6), 5 µg of pCMV-ΔMEKK1 + 10 µg of pRSV-IxBα (lanes 3 and 7), or 10 µg of pHIV-Tat (lanes 4 and 8) by electroporation. Control vectors normalized the amount of transfection DNA.

scription of the reporter 4-fold when compared to a suboptimal dose of ΔMEKK1 or JNK1 alone (Fig. 2). No synergy was observed with the control plasmid BLCAT2.

Characterization of JNK1/Rel Family Interactions—Both JNK1 and c-Rel are critical in mediating responses to T cell costimulation through the TCR and CD28 receptor. JNK1 integrates the signal transduction pathways from the TCR and CD28 receptor which trigger IL-2 secretion and proliferative responses, while c-Rel knockout mice exhibit profound defects in these same T-cell effector functions. Since MEKK1-induced transcription of these promoters is mediated by the Rel/NF-κB family and possibly JNK1, we then tested whether JNK1, which binds to its known substrates, interacted directly with c-Rel in the yeast two-hybrid system. JNK1 was subcloned into the two-hybrid vectors, pDB and pTA (to be described elsewhere), resulting in fusion to the GAL4 DNA-binding domain (pDB) and the GAL4 transcriptional-activation domain (pTA), respectively. We found that JNK1 interacted with c-Rel, but not IκBα, as indicated by β-galactosidase activity (Table I). JNK1 and c-Jun cotransformations were used as a positive control. JNK1, when fused to the DNA-binding (DB) domain or the transcriptional-activation (TA) domain of GAL4, did not cause β-galactosidase production itself. The positive interactions between c-Rel and JNK1 were seen with both pDB-JNK1 and pTA-JNK1 constructs in two different yeast strains (Table I and data not shown). The yeast two-hybrid system results demonstrate that JNK1 interacts with c-Rel in vivo.

Since JNK1 binds to c-Rel in a two-hybrid assay, we performed two-cycle immunoprecipitations (20) of [35S]methionine-labeled Jurkat T cells (Figs. 3A and B) to demonstrate JNK1-c-Rel interactions in mammalian cells. This assay identifies specific members of transcriptional complexes in vivo. Primary immunoprecipitations isolate a targeted protein from a complex which is then dissociated by boiling. Secondary immunoprecipitations of this disrupted protein complex identify members associated with the original target protein from the first immunoprecipitation. Jurkat cells were stimulated with phorbol 12-myristate 13-acetate and ionomycin during 16–20 h labeling times. Whole cell extracts were subjected to two cycles of immunoprecipitation using either anti-c-Rel or anti-JNK1 antibodies in the first cycle followed by an anti-c-Rel antibody in the second cycle. c-Rel is specifically isolated after two cycles of immunoprecipitation with the anti-c-Rel antibody, Ab265 (Fig. 3A, lane 1). To test JNK1-c-Rel interactions, JNK1 was immunoprecipitated with antibodies against different JNK1 epitopes (lanes 2–6, J 1–J 5). These JNK1-immunoprecipitated complexes were tested for the presence of c-Rel by boiling and reimmunoprecipitating with anti-c-Rel during the second cycle. c-Rel was isolated from these JNK1 complexes after the second immunoprecipitation (lanes 2–6), indicating that c-Rel associates with JNK1 in vivo.

This interaction was also seen with anti-c-Rel antibody, Ab1135 (Fig. 3B), which was generated against a different c-Rel epitope. c-Rel is detected in these lysates after immunoprecipitating first with an antibody against the NH2 terminus of JNK1 followed by Ab1135 or Ab265 (lanes 2 and 3, respec-
interaction of JNK1 with c-Rel suggests that JNK1 may phos-
tively. The c-Rel complexed with JNK1 in vivo corresponds to
the c-Rel isolated by two-cycle immunoprecipitations with
Ab265 (lane 1). These co-immunoprecipitation data, using mul-
tiple JNK1 and c-Rel antibodies, show that JNK1 associates
with c-Rel in Jurkat cells in vivo. Furthermore, the use of five
different anti-JNK1 antibodies raised against distinct JNK1
epitopes eliminates the possibility of cross-reactivity by anti-
JNK1 antibodies with c-Rel. Similarly, the use of two anti-c-Rel
epitopes eliminates the possibility of cross-reactivity by anti-
c-Rel antibodies with JNK1. Finally, the interaction is specific, since a primary
immunoprecipitation with preimmune sera did not result in
isolation of c-Rel after a second cycle of anti-c-Rel immunopre-
cipitation (Fig. 3A, lane 7). The combination of the immunopre-
cipitation and two-hybrid system data indicate that c-Rel and
JNK1 interact with each other in vivo.

JNK1 has been shown to bind to and phosphorylate the
transcription factors c-Jun, ATF2, and Elk-1. The in vivo in-
teraction of JNK1 with c-Rel suggests that JNK1 may phos-
phorylate c-Rel or other NF-κB family members. To test this
hypothesis, we performed immunocomplex kinase assays with
an anti-JNK1 antibody, Ab101, using recombinant NF-κB family
members as substrates. Ab101 specifically recognized a UV-
and anisomycin-stimulated 46-kDa protein kinase in an in-gel
kinase assay using GST-c-Jun as a substrate (Fig. 4A), indicat-
ing the antibody was not immunoprecipitating multiple kinases.
Furthermore, the antibody recognized endogenous and recom-
binant JNK1 in a Western blot (data not shown). In the immu-
nocomplex kinase assays, however, JNK1 did not phosphoryl-
ate any of the recombinant NF-κB family members tested either
alone or in combination with 1μg c-Rel (Fig. 4B and data not shown).

Since JNK1 bound c-Rel in vivo but did not phosphorylate
Rel family members in vitro, we investigated c-Rel’s ability to
compete with c-Jun and interfere with JNK1 activity. JNK1
was immunoprecipitated from anisomycin-stimulated
extracts, washed, and preincubated with either buffer, GST, or GST-c-
Rel at room temperature (Fig. 5). GST-c-Jun(1-79) was then added
and a kinase reaction was performed. When GST was
added alone (lane 2), no inhibition of c-Jun phosphorylation
was observed when compared with buffer preincubation (lane
1). However, as an equal or greater number of moles of GST-
c-Rel compared to c-Jun were added, inhibition of c-Jun phos-
phorylation was observed (lanes 4-6). Preincubation with half
the number of GST-c-Rel moles had no detectable effect (lane
3). This competition most likely results from c-Rel binding to

FIG. 3. JNK1 associates with c-Rel in vivo. 10 ng/ml phorbol
12-myristate 13-acetate + 1 μM ionomycin stimulated (16 h), 35S-Met-labeled
Jurkat cell lysate was subjected to two-
cycle immunoprecipitations (20) with an-
ti-c-Rel and anti-JNK1 antibodies. A, JNK1 associates with c-Rel. Immunopre-
cipitations (1st IP) were performed with anti-c-Rel Ab265 (ωR, lane 1), anti-
JNK1 COOH terminus (ω91, lane 2), anti-JNK1 Ab102 (ωJ-2, lane 3), anti-JNK1
Ab101 (ωJ-3, lane 4), anti-JNK1 NH2 terminus (ωJ-4, lane 5), anti-JNK1 recom-
binant protein (ωJ-5, lane 6), and normal rabbit sera (ωns, lane 7). Subsequently,
the precipitates were boiled in SDS and immunoprecipitated a second time (2nd
IP) with the anti-c-Rel Ab265 (ωR). B, immunoprecipitations (1st IP) were done
with anti-c-Rel Ab265 (ω1, lane 1), and anti-JNK1 (ωJ-4, lanes 2 and 3) followed
by boiling and re-immunoprecipitation (2nd IP) with anti-c-Rel Ab265 (ωR, lanes 1 and 3), anti-c-Rel Ab1135 (ωR, lane 2).

FIG. 4. JNK1 does not phosphorylate Rel family members in vitro. A, an inducible, 46-kDa protein kinase is recognized by anti-
JNK1 Ab101 in Jurkat cells. In-gel kinase assay (6) using Ab101 im-
munoprecipitates from untreated Jurkat cells (lane 1) or Jurkat cells
 treated with 100 J/m2 UV irradiation (lane 2) or 50 μM/ml anisomycin
(lane 3) using G-Jun(1-331) as a substrate. B, immunocomplex kinase
assays using anti-JNK1 Ab101 reveal no phosphorylation of Rel family
members in vitro. 40 μg Jurkat whole cell extract were immunopre-
cipitated with 4 μl of anti-JNK1 Ab101. The JNK1 precipitate was then
used in kinase assays with 3 μg of GST-c-Jun(1-331) (lane 1), GST-c-
Jun(1-79) (data not shown), GST-c-Rel (lane 2), GST-p50 (lane 3), or
GST-p65 (lane 4).
Functional Interaction between MEKK1/JNK1 and c-Rel Transcriptional Activation Pathways—Since (i) c-Rel bound to JNK1 in vivo, but was not phosphorylated in vitro, and (ii) the \( \kappa B \) sites in the HIV-LTR and IL-2R\( \alpha \)-chain gene promoter are activated by MEKK1 (Fig. 1) and c-Rel (22), we investigated the ability of MEKK1 and c-Rel to co-activate IL-2R\( \alpha \)-CAT and 2x\( \kappa B \)-CAT using the MEKK1 and c-Rel expression vectors. Transfection of \( \Delta \)MEKK1 or c-Rel alone activated these reporters slightly as compared to vector alone (Fig. 6, A and B). Upon cotransfection, c-Rel synergized with MEKK1 causing a 7-10-fold induction of CAT activity from both reporters (Fig. 6, A and B). Furthermore, to demonstrate that this synergy was not specific to Jurkat cells, the same transient transfection experiment was performed in 293T cells. As seen in Jurkat cells (Fig. 6, A and B), neither MEKK1 nor c-Rel alone activated \( \kappa B \) sites significantly using the HIV-CAT reporter (Fig. 6C) in 293T cells. However, synergy was observed when both c-Rel and MEKK1 were cotransfected (Fig. 6C) resulting in a 6-7-fold induction of CAT activity from the reporter. Thus, the MEKK1/c-Rel synergy is reproducible in different cell lines. No transcriptional activity was observed using the IL-2R\( \alpha \)-CAT or HIV(\( \kappa B \))-CAT, and BLCAT2 controls.

To demonstrate that MEKK1 was activating JNK1 specifically in this transfection system, immunocomplex kinase assays were performed. JNK1, p38, and ERK2 were immunoprecipitated from empty vector and \( \Delta \)MEKK1-transfected 293T cells (Fig. 7). JNK1 was activated 50-fold in the \( \Delta \)MEKK1-transfected cell lysates (lane 2) compared to control transfected lysates (lane 1), while ERK2 and p38 activity remained relatively unchanged (compare lanes 4 and 6 with lanes 3 and 5, respectively). These data show that MEKK1 activates JNK1 and not the other MAPKs, ERK2 and p38, in transient transfection assays. Furthermore, the transfection and kinase results suggest a synergistic role for MEKK1-activated JNK1 and c-Rel in \( \Delta \)MEKK1-mediated activation of the \( \kappa B \) elements.

**FIG. 5.** c-Rel blocks JNK1 phosphorylation of c-Jun in vitro. JNK1 was immunoprecipitated from anisomycin lysates, washed, and preincubated with buffer (lane 1), GST (lane 2), or GST-c-Rel(2-587) (lanes 3-6) for 35 min at room temperature. GST-c-Jun(1-79) was added and kinase reactions performed. \( n \times \) equals the number of moles of GST-c-Rel added relative to GST-c-Jun.

**DISCUSSION**

c-Rel and JNK1 are critical molecules involved in CD28 signaling and T-cell activation. Both are activated by CD28 costimulation leading to c-Rel-mediated IL-2 transcriptional activation and IL-2 production. This study provides evidence that the MEKK1 signaling module and c-Rel interact in T cells. The MEKK1 module drives transcription from the HIV-LTR and IL-2R\( \alpha \) reporters in a \( \kappa B \)-dependent manner and syner-
...with c-Rel in activating transcription from these reporters. JNK1 involvement in mediating MEKK1-activated NF-κB transcription is suggested by the fact that JNK1, not ERK2 nor p38, is activated in MEKK1-transfected cell lysate (Fig. 7). Cotransfection of a dominant negative JNK1 construct with MEKK1 would solidify JNK1’s role in this MEKK1-activated NF-κB pathway, but attempts thus far have failed possibly due to an inefficient inhibitory effect of the JNK1 mutant2 and/or high endogenous JNK1 activity in Jurkat cells (data not shown). Nevertheless, this study provides the first demonstration of MEKK1-activated NF-κB transcription.

Additional evidence for interaction between the JNK1 and NF-κB activation pathways is demonstrated through binding studies. JNK1 bound directly to c-Rel in vivo in a yeast two-hybrid assay (Table I) and associated with c-Rel in vivo in Jurkat cells as shown by two-cycle immunoprecipitations (Fig. 3). Furthermore, GST-c-Rel blocked JNK1 phosphorylation of a truncated GST-c-jun substrate in vitro in immunocomplex kinase assays (Fig. 5). The results from these three assays indicate that c-Rel binds to JNK1 in vivo and that c-Rel binds to the activated form of the JNK1 protein kinase in vitro. As JNK1 binds and phosphorylates other transcription factors like c-jun and ATF2, NF-κB phosphorylation may be the result of the JNK1-c-Rel interaction. However, no phosphorylation of Rel/NF-κB family members was observed in vitro (Fig. 4 and data not shown). While the lack of phosphorylation in vitro does not rule out Rel family members as substrates, other c-Rel-associated proteins may be involved in vivo targets for JNK1. For example, c-Rel may serve as a docking or stabilizing molecule for the JNK1 protein kinase and cause enhanced phosphorylation of or access to c-Rel-associated JNK1 substrates. As many common agents activate the MEKK1 module and c-Rel, this interaction between JNK1 and c-Rel would potentiate the signaling effects of these proteins as suggested by the observed synergy between c-Rel and MEKK1 (Fig. 6). Our finding of the physical association of c-Rel and JNK1 in T cells, coupled with their involvement in common T cell signaling cascade, will lead to the discovery of other novel JNK1 substrates that are important in T cell signal transduction. This lays the foundation to investigate the cross-talk between JNK1 and c-Rel in signaling cascades.

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Fig. 7. Preferential JNK1 activation by transfected MEKK1. 5 μg of pEE-CMV or 5 μg of pCMV-ΔMEKK1 was transfected into 293T cells, and JNK1, p38, and ERK2 activation was measured by immunocomplex kinase assays. 2.5 μg of GST-c-jun(1–79) was used as a JNK1 substrate and 5 μg of myelin basic protein was used as a p38 or ERK2 substrate.