Tumor Necrosis Factor (TNF) Receptor 1 Signaling Downstream of TNF Receptor-associated Factor 2

NUCLEAR FACTOR κB (NFκB)-INDUCING KINASE REQUIREMENT FOR ACTIVATION OF ACTIVATING PROTEIN 1 AND NFκB BUT NOT OF c-Jun N-TERMINAL KINASE/STRESS-ACTIVATED PROTEIN KINASE "

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Like other members of the tumor necrosis factor (TNF) receptor family, p55 TNF receptor 1 (TNF-R1) lacks intrinsic signaling capacity and transduces signals by recruiting associating molecules. The TNF-R1 associated death domain protein interacts with the p55 TNF-R1 cytoplasmic domain and recruits the Fas-associated death domain protein (which directly activates the apoptotic proteases), the protein kinase receptor interacting protein, and TNF receptor-associated factor 2 (TRA2F). TRAF2 has previously been demonstrated to activate both transcription factor nuclear factor κB (NFκB) and the c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) pathway, which in turn stimulates transcription factor activating protein 1 (AP1) mainly via phosphorylation of the c-Jun component. We have investigated the signaling properties of NFκB-inducing kinase (NIK), a TRAF2-associated protein kinase that mediates NFκB induction. NIK was found to be unable to activate JNK/SAPK, mitogen-activated protein kinase, or p38 kinase. Moreover, NIK was not required for JNK/SAPK activation by TNF-R1, thus representing the first TNF-R1 complex component to disent NFκB and the JNK/SAPK pathways. Despite being unable to activate JNK/SAPK and mitogen-activated protein kinase, NIK strongly activated AP1 and was required for TNF-R1-induced AP1 activation. Therefore, NIK links TNF-R1 to a novel, JNK/SAPK-independent, AP1 activation pathway.

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1 The abbreviations used are: TNF, tumor necrosis factor; TNF-R, TNF receptor; TRADD, TNF receptor-associated death domain protein; FADD, Fas-associated death domain protein; TRAF2, TNF receptor-associated factor 2; JNK, c-Jun N-terminal kinase; SAPK, stress-activated protein kinase; NFκB, nuclear factor κB; IKK, NFκB-inducing kinase; MAPK, mitogen-activated protein kinase; AP1, activating protein 1; PCR, polymerase chain reaction; aa, amino acid(s); DTT, dithiothreitol; HR, human recombinant; CAT, chloramphenicol acetyltransferase; MEKK, mitogen-activated/extracellular response kinase kinase kinase; SEK, stress-activated protein kinase kinase; JNKK, JNK kinase.

EXPERIMENTAL PROCEDURES

Expression Vectors—Full-length human NIK cDNA was PCR amplified from a human placental cDNA library using a mixture of Tag and two polymerases (Boehringer). The primers used were NIK1 (5’-TCGGTACCATGGGCTGTTCTC-3’) and NIK2 (5’-TCTCTCTGGGCTGTTCTC-3’); the PCR fragment was digested with NheI and XhoI and cloned in pcDNA3-HA. pcDNA3-HA was constructed by insertion of a BagI/BamHI fragment from pActII (CLONTECH) into the BamHI site of pcDNA3 (Invitrogen, Inc). NIK1234 (deletion of aa 1–334) was constructed by PCR amplification of full-length NIK using the primers 1234 (5’- TTGGTACCATGGGCTGTTCTC-3’) and NIK2. NIKΔ101 (deletion of aa 1–623) was amplified using the primers 2101 (5’- TTGGTACCATGGGCTGTTCTC-3’) and NIK2. Both PCR fragments were cloned in pcDNA3-HA as above. NIKKR was obtained by two-step PCR using the mutagenic primers KR5 (5’-CAGTGGCGCTCGTCAAGGAGTCCGGCTGTTCTC-3’) and KRA5 (5’-CAGCAGCAGGCTCGTCAAGGAGTCCGGCTGTTCTC-3’). HA-p65SAKpCDNA3 and HA-SEKALpMT2 (gifts of J. R. Comment
Similarly to JNK/SAPK, p38 activation by TNF depends on NIK, and consistent with a requirement for NIK in TNF-induced NF-κB activation, mutation at aa 429) abolish NF-κB activation, whereas the deletion of the catalytic domain (NIKΔ2101) or its inactivation (Lys → Arg mutation at aa 429) abolish NF-κB activation (Fig. 1, A and B). Deletion of the N-terminal (putatively regulatory) domain of NIK (NIKΔ1234) does not apparently affect NF-κB activation, whereas the deletion of the catalytic domain (NIKΔ2101) or its inactivation (Lys → Arg mutation at aa 429) abolish NF-κB activation (Fig. 1, B). Consistent with a requirement for NIK in TNF-induced NF-κB activation, overexpression of a C-terminal NIK fragment (NIKΔ2101), which binds TRAF2 and presumably blocks the recruitment of endogenous NIK and/or titrates downstream effectors (23), significantly impairs the induction of NF-κB by either TNF treatment or overexpression of TNF-R1 complex components (Fig. 1C). These data indicate that NIK is required for the activation of NF-κB by TNF-R1/TRAP2 in different cell types.

Because TRAF2 overexpression is sufficient to activate both NF-κB and JNK/SAPK (13–16), we examined whether NIK is able to activate JNK/SAPK as well. NIK was cotransfected in HeLa and 293 cells together with a hemagglutinin (HA)-tagged SAPKα expression vector, and the activity of exogenous transfected SAPKα was assayed 36–48 h after transfection. In 293 cells, wherein TRAF2 usually gives the highest activation of SAPKα/JNK, neither NIK nor NIKΔ1234 (which are both efficient NF-κB activators) were able to elevate JNK/SAPK activity over the baseline. In a similar manner, we were unable to detect any JNK/SAPK activation by NIK in HeLa cells (Fig. 2, A and B). Similarly to NIK/SAPK, p38 activation by TNF depends on TRAF2 (14, 21) but is not dependent on NIK (Fig. 2C).

Apart from inducing a prolonged activation of JNK/SAPK and p38, TNF-R1 engagement provokes a mild and transient activation of the mitogen-activated protein kinase (MAPK), whose biological role has not been defined (26, 27). MAPK activation by TNF may depend on a TNF-R1 domain that is distinct from the TRADD interaction domain and that interacts with a recently identified protein known as FAN (28). Consistent with MAPK activation being a TRADD-independent function, neither TRAF2 or NIK were able to activate MAPK in the cells tested (Fig. 2D).

The effects of the dominant negative NIK mutant (NIKΔ2101) on JNK/SAPK activation by TNF-R1 were next evaluated. The expression of Δ2101 at levels that gave maximal inhibition of NF-κB induction (Fig. 1C) did not impair the ability

**RESULTS AND DISCUSSION**

In both 293 and HeLa cells, TRADD, TRAF2, and NIK are able to induce a strong NF-κB activation (NIK being the strongest activator) (Fig. 1, A and B). Deletion of the N-terminal (putatively regulatory) domain of NIK (NIKΔ1234) does not apparently affect NF-κB activation, whereas the deletion of the catalytic domain (NIKΔ2101) or its inactivation (Lys → Arg mutation at aa 429) abolish NF-κB activation (Fig. 1, B). Consistent with a requirement for NIK in TNF-induced NF-κB activation, overexpression of a C-terminal NIK fragment (NIKΔ2101), which binds TRAF2 and presumably blocks the recruitment of endogenous NIK and/or titrates downstream effectors (23), significantly impairs the induction of NF-κB by either TNF treatment or overexpression of TNF-R1 complex components (Fig. 1C). These data indicate that NIK is required for the activation of NF-κB by TNF-R1/TRAP2 in different cell types.

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of either TNF or TRAF2 to activate JNK/SAPK (Fig. 2E). Therefore, when the NIK pathway is blocked by expression of dominant negative NIK, both receptor cross-linking and overexpressed TRAF2 still activate JNK/SAPK. Taken together our results suggest that: (i) NIK is neither sufficient nor required for JNK/SAPK activation by TNF-R1/TRAF2; (ii) the bifurcation between the NFkB pathway and the JNK/SAPK pathway occurs immediately downstream of TRAF2; and (iii) dominant negative NIK does not disrupt the TNF-R1 complex nonspecifically. Therefore, NIK disrupts the TRAF2 pathway leading to NFkB activation from the pathway leading to JNK/SAPK activation; this suggests that the
ability of TNF-R1/TRAF2 to activate JNK/SAPK must depend on a different TRAF2-interacting protein. One possible candidate is represented by MEKK1, a kinase that phosphorylates and activates SEK/JNKK, which in turn phosphorylates JNK/SAPK (29–32). However, we have been unable to detect a physical interaction between TRAF2 and MEKK1. Therefore, the evidence for a role of MEKK1 in TNF-R1 signaling is indirect and arises from the ability of catalytically inactive MEKK1 (MEKK1-KM) to block TNF-R1/TRAF2-induced activation of SAPK/JNK (14); at this point we cannot exclude the possibility that SAPK/JNK activation by TNF-R1/TRAF2 depends on a putative MEKK1-related protein whose activity is inhibited by MEKK1-KM overexpression.

The prolonged activation of JNK/SAPK by TNF and the consequent phosphorylation and activation of the c-Jun transcriptional activation domain correlate with the sustained induction of AP1-dependent genes (33, 34). AP1 is composed of proteins of the Jun and Fos families that associate to form a variety of homo- and heterodimers that bind to a common recognition element known as either the tetradecanoic parabol acetate-response element or the AP1 binding site (35); the variety of homo- and heterodimers that bind to a common recognition element known as either the tetradecanoic parabol acetate-response element or the AP1 binding site (35); the

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