IKKγ Serves as a Docking Subunit of the IκB Kinase (IKK) and Mediates Interaction of IKK with the Human T-cell Leukemia Virus Tax Protein*

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The tax gene product of human T-cell leukemia virus type I induces activation of transcription factor NF-κB, which contributes to deregulated expression of various cellular genes. Tax expression triggers persistent phosphorylation and degradation of the NF-κB inhibitory proteins IκBα and IκBβ, resulting in constitutive nuclear expression of NF-κB. Recent studies demonstrate that Tax activates the IκB kinase (IKK), although the underlying mechanism remains unclear. In this report, we show that Tax physically interacts with a regulatory component of the IKK complex, the NF-κB essential modulator or IKKγ (NEMO/IKKγ). This molecular interaction appears to be important for recruiting Tax to the IKK catalytic subunits, IKKα and IKKβ. Expression of NEMO/IKKγ greatly promotes binding of Tax to IKKα and IKKβ and stimulates Tax-mediated IKK activation. Interestingly, a mutant form of Tax defective in IKK activation exhibited a markedly diminished level of NEMO/IKKγ association. These findings suggest that the physical interaction of Tax with NEMO/IKKγ may play an important role in Tax-mediated IKK activation.

The type I human T-cell leukemia virus (HTLV-I)1 transforms human T-cells, which is associated with the development of an acute T-cell malignancy termed adult T-cell leukemia (1). HTLV-I encodes a regulatory protein, Tax, which plays a central role in the induction of host cell transformation (1). Tax alters the expression of a large number of cellular genes involved in cell growth and survival, which appears to contribute to the oncogenic activity of this viral protein (2, 3). Lacking DNA binding activity, Tax induces the target genes indirectly by modulating the activity of specific host transcription factors (3, 4). Induction of many cellular genes by Tax is mediated through the transcription factor NF-κB (3), a key regulator of genes involved in cell activation, proliferation, and survival (for recent reviews, see Refs. 5–7). Inducing T-cells, as well as most other cell types, NF-κB is sequestered as an inactive precursor by association with specific inhibitors, including IκBα, IκBβ, and related proteins (8). Induction of NF-κB nuclear expression by cytokines and T-cell mitogens is mediated by activation of a multisubunit IκB kinase (IKK) (9–11). The IKK complex is composed of two catalytic subunits, IKKα (12–14) and IKKβ (14–16), and a noncatalytic subunit termed IKKγ (also named NF-κB essential modulator (NEMO) and IKK-associated protein 1, hereafter called NEMO/IKKγ) (17–19). Additional proteins have been shown to be physically associated with the IKK complex, which include MEK kinase 1 (MEKK1), NF-κB-inducing kinase (NIK) (12, 14, 20), and IKK complex-associated protein (21). Upon activation, IKK phosphorylates IκBα and IκBβ at two regulatory N-terminal serine residues, causing rapid ubiquitination and proteolysis of these NF-κB inhibitors, which allows the released NF-κB to enter the nucleus and activate target genes (10, 11).

We and others have shown recently that the IKK is constitutively activated in HTLV-I-infected or Tax-expressing T-cells (22–25). This action of Tax results in persistent degradation of IκBα and IκBβ and constitutive nuclear expression of NF-κB (26–30). Tax has been shown to induce the catalytic activity of both IKKα and IKKβ, although the underlying mechanism remains unclear (17). In this paper, we show that Tax physically interacts with NEMO/IKKγ, which facilitates the recruitment of Tax to the catalytic subunits IKKα and IKKβ and promotes Tax-mediated activation of IKK.

MATERIALS AND METHODS

Plasmids and Antibodies—The pcDNA-HA vector was constructed by inserting a copy of the influenza hemagglutinin (HA) epitope tag (YPYDVPDYA) together with the translation initiation codon (ATG) into the mammalian expression vector pcDNA3.1 (Invitrogen). To generate pcDNA-HA-NEMO/IKKγ, the murine NEMO cDNA (provided by Drs. S. Yamaoka and A. Israéli (17)) was subcloned into the pcDNA-HA vector downstream of the HA tag. Truncation mutants of NEMO/IKKγ were generated by restriction digestion and designated by the specific amino acid residues retained in the mutant proteins. For example, NEMO/IKKγ(1–312) contains the N-terminal 312 amino acids. pcCMV4-HA-NIK was generated by inserting HA-tagged human NIK cDNA (provided by Dr. David Wallach (20)) in the pcCMV4 vector (31). pcCMV4-HA-IκBα was described previously (32). The pcDNA-HA-IKKα, pcDNA-HA-IKKβ, and pcDNA-HA-MEKK1 were provided by Dr. M. Karin. The pcCMV4-Tax, pcCMV4-TaxM22, and pcCMV4-TaxM47 were provided by Dr. W. C. Greene (33). The anti-HA and anti-IKK antibody clones were from Roche Molecular Biochemicals and Immunex Corp., respectively. The anti-Tax monoclonal antibody was prepared from a hybridoma (168B17-46-34) provided by the AIDS Research and Reference Program, NIAID, National Institutes of Health. All the other antibodies were purchased from Santa Cruz Biotechnology, Inc.

Immunoprecipitation (IP) and Immunoblotting Assays—Human 293 kidney carcinoma cells were seeded in 0.1% gelatin-treated six-well plates (1 × 10⁵ cells/well) and transfected using DEAE-dextran with the indicated cDNA expression vectors. The DNA amounts used for the transfections were normalized based on the expression efficiency of each of the expression vectors: 50 ng for IKKα, IKKβ, MEKK1, and NIK; 50–200 ng for NEMO/IKKγ and its truncation mutants; 0.5 μg for Tax and its mutants. After 48 h, recipient cells were lysed in RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiotreitol, 0.01 volume of a protease inhibitor mixture (34)). The RIPA buffer was also used to prepare whole-cell extracts from the HTLV-I-infected SLB-1 cells (24). IP was performed

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1 The abbreviations used are: HTLV-I, human T-cell leukemia virus type I; IKK, IκB kinase; NEMO, NF-κB essential modulator; MEKK, mitogen-activated protein kinase/extracellular signal-related kinase kinase; MEKK1, MEKK kinase 1; NIK, NF-κB-inducing kinase; DN-NIK, dominant-negative NIK; IP, immunoprecipitation; HA, hemagglutinin; RIPA, radioimmune precipitation buffer.

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as described previously (34), and the precipitated proteins were analyzed by SDS-polyacrylamide gel electrophoresis followed by immunoblotting. For immunoblotting analyses of IgH phosphorylation, 293 cells were transfected in 24-well plates. Whole-cell extracts were prepared in ELB buffer (34) supplemented with phosphatase inhibitors and analyzed by immunoblotting (34).

RESULTS

NEMO/IKKγ Serves as a Primary Target of Tax in the IKK Complex—Prior studies suggest that Tax interacts with the IKK catalytic subunits IKKα and IKKβ (23) as well as the upstream kinase MEKK1 (22). However, since the IKK components are present in a large complex in intact cells, it remains unknown which component serves as the primary target of Tax. To address this question, co-IP was performed using a high stringency binding buffer (RIPA) to assess the relative binding affinity of Tax with the different molecular components of the IKK complex. Tax was transiently expressed in 293 cells either alone or together with each of the known IKK components, including IKKα, IKKβ, NEMO/IKKγ, MEKK1, and NIK, all of which were tagged with the HA antigenic epitope. In the absence of an IKK component, Tax was not precipitated by the anti-HA antibody (Fig. 1A, lane 3), although this protein was readily precipitated by the anti-Tax antibody (lane 2), demonstrating the specificity of the antibodies. Under these conditions, a small amount of Tax was coprecipitated with IKKα and NIK (lanes 4 and 8). Coprecipitation of Tax with IKKβ and MEKK1 was also detected after an extended exposure time (data not shown). Importantly, the IP assays revealed a remarkably higher amount of Tax coprecipitated with NEMO/IKKγ (Fig. 1A, lane 6). Thus, while Tax may weakly bind to various components of the IKK complex, NEMO/IKKγ appears to be a major target of this viral transactivator protein. To address the physiological relevance of this finding, we examined the interaction between Tax and NEMO/IKKγ in HTLV-I-infected T-cells. As expected, Tax was readily detected from the immune complex precipitated by the anti-Tax antibody (Fig. 1C, lane 2), but not from that precipitated by a preimmune serum (lane 1). More importantly, a significant amount of Tax was coprecipitated with IKKγ by an IKKγ-specific antibody (lane 3). These findings clearly demonstrated that Tax stably binds to NEMO/IKKγ in both transfected and HTLV-I-infected cells.

Different Sequences of NEMO/IKKγ Are Involved in Binding to Tax and IKKα—NEMO/IKKγ is known to form a stable complex with IKKα and IKKβ in vitro (17, 18). To map the regions of NEMO/IKKγ involved in binding to the IKK catalytic subunits and Tax, progressive truncations were generated from both the N and C termini of NEMO/IKKγ. Deletion of the N-terminal 110 amino acids of NEMO/IKKγ did not affect its binding to IKKα (Fig. 2A, lane 3) or Tax (lane 10). Deletion of up to 292 amino acids from the C terminus also did not affect the IKKα association (lanes 4–6), although further removal of 8 amino acids generated a mutant (1–112) that no longer bound IKKα (lane 7). In contrast to that seen with IKKα, Tax inter-
Fig. 2. NEMO/IKKγ promotes interaction of Tax with IKKα and IKKβ and stimulates Tax-mediated IKKβ activation. A. 293 cells were transfected with Tax together with HA-IKKα (lanes 2-4) or HA-IKKβ (lanes 5-7), either in the absence (−) or presence of the full-length (FL) or C-terminal truncated (1-312) NEMO/IKKγ. Lane 1 was a control transfected with an empty vector. Cell extracts were subjected to IP with anti-Tax followed by detection of the coprecipitated IKKs by Immunoblotting (IB) with anti-HA. B. 293 cells were transfected with HA-IKKβ (25 ng) together with either an empty vector (lane 1) or the indicated amounts of Tax and HA-tagged NEMO/IKKγ constructs. All the cells also received the HA-IκBα expression vector (0.12 µg). Cell extracts were subjected to immunoblotting using anti-IκBα (upper panel), anti-HA (middle panel), or anti-Tax (lower panel). Basal and phosphorylated (P) forms of IκBα and IKKβ, the full-length (FL) and truncated (1-312) NEMO/IKKγ, as well as Tax, are indicated. Note that IκBα and IκBα-P were detected by both the anti-IκBα (upper panel) and anti-HA (lower panel) antibodies. C. 293 cells were transfected with IKKβ together with an empty vector (lane 1) or full-length NEMO/IKKγ (lane 2) in the absence of Tax, and the IκBα and NEMO/IKKγ proteins were analyzed by immunoblotting using anti-IκBα (upper panel) or anti-HA (lower panel). IKKβ is not shown in the figure. D. 293 cells were transfected with HA-tagged IκBα and the indicated cDNA expression vectors (DNA amounts were as described for lane 5 of B). The cells were also transfected with the indicated amounts of a dominant-negative NIK (DN-NIK). The transfected IκBα and Tax proteins were analyzed by immunoblotting using anti-HA (upper panel) and anti-Tax (lower panel), respectively.

action with NEMO/IKKγ required the C-terminal sequences of NEMO/IKKγ. Removal of 100 amino acids from this end of the molecule significantly reduced, although not abolished, its interaction with Tax (Fig. 2A, lane 11). This low level binding activity was also detected between Tax and an IKKγ mutant lacking its C-terminal 157 amino acids (lane 12). However, further deletion of 135 or more amino acids from this end generated IKKγ mutants (1-120 and 1-112), which completely lost Tax-binding activity (lanes 13 and 14). Of note, IKKγ(1-120) still retained its IKKγ binding activity. Thus, while the N-terminal 120 amino acids of NEMO/IKKγ are sufficient for binding to IKKγ, the C-terminal region is required for strong interaction with Tax.

NEMO/IKKγ Facilitates Binding of Tax to the Catalytic Subunits of IKK and Promotes Tax-mediated IKK Activation—To assess the role of the interaction between Tax and NEMO/IKKγ in IKK activation, we examined the effect of NEMO/IKKγ on Tax interaction with the catalytic subunits of IKK and on Tax-mediated IKK activation. For these studies, Tax was coexpressed with HA-tagged IKKα for IKKβ together with either the full-length NEMO/IKKγ or its C-terminal truncation mutant, 1-312. We found that the low level interaction of IKKα and IKKβ with Tax could be more readily detected when the IP was performed using the anti-Tax antibody (Fig. 3A, lanes 2 and 5). More importantly, the interaction of Tax with both IKKα and IKKβ was remarkably enhanced by the full-length NEMO/IKKγ (lanes 3 and 6). Parallel immunoblotting assays showed that NEMO/IKKγ did not affect the expression level of the IKKs (data not shown). Furthermore, this stimulatory effect was only weakly detected with the NEMO/IKKγ-(1-312) (lanes 4 and 7), a C-terminal truncation mutant exhibiting reduced affinity in Tax binding (see Fig. 2A).

We then examined the effect of NEMO/IKKγ on Tax-mediated IKK activation by measuring the in vivo phosphorylation of its substrate IκBα. In this regard, the in vivo IκBα phosphorylation could be detected by immunoblotting since the phosphorylated IκBα migrates more slowly on SDS gels (27, 35-37). As we reported previously (24), expression of IKKβ alone or together with Tax in 293 cells did not induce appreciable IκBα phosphorylation (Fig. 3B, upper panel, lanes 1 and 2). However, when these cells were cotransfected with the full-length NEMO/IKKγ, the IKKβ kinase activity was markedly induced, resulting in the potent phosphorylation of the substrate IκBα (upper panel, lanes 5 and 6, IκBα-P). Degradation of IκBα became evident when a higher dose of NEMO/IKKγ was used (lane 6, upper panel). We also observed that Tax induces the autophosphorylation of IKKβ in the presence of NEMO/IKKγ (middle panel, lanes 5 and 6, IKKβ-P). These functional effects were not significantly detected with the NEMO/IKKγ-(1-312) (lanes 3 and 4). Furthermore, NEMO/IKKγ was unable to activate IKKβ in the absence of Tax (Fig. 3C). Together, these results strongly suggest that NEMO/IKKγ promotes association of Tax with the IKK catalytic subunits and stimulates Tax-mediated IKK activation.

To assess the role of upstream kinases in the IKKγ-dependent induction of IKKβ by Tax, we examined the effect of a dominant-negative NIK (DN-NIK) on the in vivo phosphorylation of IκBα triggered by Tax (Fig. 3D). As expected, phosphorylated form of IκBα was readily detected in cells transfected with IKKβ together with NEMO/IKKγ and Tax (lane 1). Cotransfection of the DN-NIK led to a partial inhibition of the IκBα phosphorylation. Thus, it is likely that Tax-mediated activation of IKK may involve both its physical interaction with IKK and the participation of upstream kinases.

A Mutant Form of Tax, Defective in IKK Binding, Is Inefficient in NEMO/IKKγ Binding—To further assess the functional importance of Tax interaction with NEMO/IKKγ, we examined the ability of two well characterized Tax mutants, M22 and M47 (33), to interact with NEMO/IKKγ. The M22 is defective in IKK activation and induction of NF-κB nuclear expression, while M47 remains competent in these functions (22-25). Interestingly, co-IP assays revealed that while the wild type Tax and M47 both strongly interacted with NEMO/IKKγ (Fig. 4, upper panel, lanes 2 and 4), only a marginal NEMO/IKKγ binding activity was detected with M22 (lane 3).
Thus, the physical interaction of Tax with NEMO/IKKγ is well correlated with its ability to activate the IKK catalytic subunits.

DISCUSSION

HTLV-I-encoded Tax protein is a potent activator of the cellular transcription factor NF-κB. Tax activation of NF-κB involves phosphorylation and degradation of the NF-κB inhibitory proteins IκBα and IκBβ (26–30). Tax activates the catalytic activity of IKK in both transiently transfected and HTLV-I-transformed cells (22–25). The mechanism by which Tax activates IKK remains elusive. A recent study suggests that the NEMO/IKKγ subunit of IKK is essential for Tax-mediated NF-κB activation, although it is unclear how this noncatalytic signaling protein participates in Tax activation of IKK (17).

Our current data demonstrate that NEMO/IKKγ physically interacts with Tax in both transfected 293 cells and HTLV-I-infected T-cells. Under stringent IP conditions, NEMO/IKKγ exhibited markedly higher Tax binding activity than various other IKK components (Fig. 1). The functional importance of this physical interaction in Tax-mediated activation of IKK is supported by the experiments performed with Tax mutants (Fig. 4). The M22 mutant, known to be defective in IKK activation (22–25), the C-terminal region of NEMO/IKKγ is required for its strong binding to Tax, whereas the N-terminal region of NEMO/IKKγ mediates interaction with IKKα. Although further studies are required to demonstrate that NEMO/IKKγ can form a heterotrimer with Tax and IKKα (or IKKβ), our current data show that NEMO/IKKγ indeed promotes the binding of Tax to IKKα and IKKβ as well as stimulates the Tax-mediated IKK activation (Fig. 3). Furthermore, these functions were not significantly detected with a NEMO/IKKγ C-terminal truncation mutant, 1–312, which exhibits markedly reduced Tax binding activity.

It remains to be further studied how the recruitment of Tax to IKK complex triggers IKK activation. A recent study suggests that Tax also physically interacts with MEKK1 and activates the catalytic activity of this IKK-activating kinase (22). Thus, an intriguing model is that Tax recruits upstream kinases, such as MEKK1, to the IKK complex, thus triggering phosphorylation-dependent IKK activation. However, interaction of Tax with MEKK1 appears to be relatively weak under the stringent IP conditions used in this study (Fig. 1). We have shown previously that NIK is functionally involved in Tax activation of IKK, although this kinase also does not strongly bind to Tax. It remains to be examined whether the weak interaction of Tax with MEKK1 or NIK is sufficient to recruit these upstream kinases to IKK. Nevertheless, we have shown that the IKKy-dependent IKK activation by Tax is at least partially sensitive to DN-NIK. This finding suggests that Tax activation of IKK may involve both Tax/IKK physical interaction and activation or recruitment of upstream kinases.

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REFERENCES
1. Feuer, G., and Chen, I. S. Y. (1992) Biochim. Biophys. Acta 1114, 223–233
2. Green, P. L., and Chen, I. S. Y. (1996) FASEB J. 4, 169–175
3. Smith, M. R., and Greene, W. C. (1991) J. Clin. Invest. 87, 761–766
4. Greene, W. C., Bohmlein, E., and Ballard, D. W. (1989) Immunois. Today 10, 272–278
5. Siebenlist, U. (1997) Biochim. Biophys. Acta 1332, R7–R13
6. Gerondakis, S., Grumont, R., Rourke, I., and Grossmann, M. (1998) Curr. Opin. Immunol. 10, 353–359
7. Ghosh, S., May, M. J., and Kopp, E. B. (1998) Annu. Rev. Immunol. 16, 225–260
8. Baldwin, A. S., Jr. (1996) Annu. Rev. Immunol. 14, 649–683
9. Baldwin, A. S., Jr. (1996) Annu. Rev. Immunol. 14, 649–683
10. Stancovski, I., and Baltimore, D. (1997) Cell 91, 299–302
11. Karin, M. (1998) Cancer J. Sci. Am. 4, Suppl. 1, S92–S99
12. Rougeot, C. H., Song, H. Y., Cao, Z., and Rothwarf, D. M. (1997) Cell 90, 373–383
13. DiDonato, J. A., Hayakawa, M., Rothwarf, D. M., Zandi, E., and Karin, M. (1997) Nature 388, 548–554
14. Mercurio, F., Zhu, H., Murray, B. W., Shevchenko, A., Bennett, B. L., Li, J. W., Young, D. B., Barbose, M., Mann, M., Manning, A., and Rao, A. (1997) Science 278, 860–865
15. Wornowicz, J. D., Gao, X., Cao, Z., Rothie, M., and Goedelev, D. V. (1997) Science 278, 866–869
16. Zandi, E., Rothwarf, D. M., Delhase, M., Hayakawa, M., and Karin, M. (1997) Cell 91, 243–252
17. Yamaoka, S., Courtris, G., Bessia, C., Whitehead, S. T., Weil, R., Agou, F., Kirk, H. E., Kay, R. J., and Israel, A. (1998) Cell 93, 1231–1240
18. Rothwarf, D. M., Zandi, E., Natoli, G., and Karin, M. (1998) Nature 373, 297–300
19. Mercurio, F., Murray, B. W., Shevchenko, A., Bennett, B. L., Young, D. B., Li, J. W., Pascual, G., Motiwala, A., Zha, H., Mann, M., and Manning, A. M. (1999) Mol. Cell. Biol. 19, 1526–1538
20. Malinini, N. L., Boldin, M. P., Kovalenko, A. V., and Wallach, D. (1997) Nature 385, 540–544
21. Cohen, L., Henzel, W. J., and Baeuerle, P. A. (1998) Nature 17, 292–296
22. Yin, M. J., Christersson, L. B., Yamamoto, Y., Kwak, Y. T., Xu, R., Mercurio, F., Barbose, M., Cobb, M. H., and Gaynor, R. B. (1998) Cell 93, 875–884
23. Chu, Z.-L., DiDonato, J. A., Hawiger, J., and Ballard, D. W. (1998) J. Biol. Chem. 273, 15891–15894
24. Uehling, D. T., Cristofanilli, B., Kavanagh, J., Darzynkiewicz, Z., Young, D. B., Barbose, M., Cobb, M. H., and Gaynor, R. B. (1998) Cell 93, 875–884
25. Geletu, A., Ferrero, S., Lin, X., Xu, M., Cunningham, E. T., Jr., Grant, M., Connelly, M. A., Hanbo, E., Marcu, K. B., and Greene, W. C. (1998) Mol. Cell. Biol. 18, 5157–5165
26. Sun, S.-C., Elwood, J., Béraud, C., and Greene, W. C. (1994) Mol. Cell. Biol. 14, 7577–7584
27. Brockman, J. A., Scherer, D. C., McKinsey, T. A., Hall, S. M. Q., Xi, L., Lee, W. Y., and Ballard, D. A. (1995) Mol. Cell. Biol. 15, 2809–2818
28. Lacoste, J., Petropoulos, L., Pe´pin, N., and Hiscott, J. (1995) Mol. Cell. Biol. 15, 564–569
29. Good, L., and Sun, S.-C. (1996) J. Virol. 70, 2730–2735
30. McKinsey, T. A., Brockman, J. A., Scherer, D. C., Al-Murrani, S. W., Green, P. L., and Ballard, W. L. (1996) Mol. Cell. Biol. 16, 2083–2090
31. Andersson, S., Davis, D., Dahlback, H., Jornvall, H., and Russell, K. W. (1989) J. Biol. Chem. 264, 8222–8229
32. Good, L., Magirwar, S. B., Kealiher, A., Uehli, M., and Sun, S.-C. (1996) Biochem. Biophys. Res. Commun. 233, 123–128
33. Smith, M. R., and Greene, W. C. (1990) Genes Dev. 4, 1875–1885
34. Ganchi, P. A., Sun, S.-C., Greene, W. C., and Ballard, D. W. (1992) Mol. Biol. Cell. 3, 1339–1352
35. Brown, K., Gersterberger, S., Carlsson, L., Francisco, G., and Siebenlist, U. (1995) Science 267, 1485–1488
36. Sun, S.-C., Magirwar, S. B., and Harhaj, E. (1995) J. Biol. Chem. 270, 18347–18351
37. Sun, S.-C., Elwood, J., and Greene, W. C. (1996) Mol. Cell. Biol. 16, 1058–1065