Complementation Cloning of NEMO, a Component of the IkB Kinase Complex Essential for NF-kB Activation

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Summary

We have characterized a flat cellular variant of HTLV-1 Tax-transformed rat fibroblasts, 5R, which is unresponsive to all tested NF-kB activating stimuli, and we report here its genetic complementation. The recovered full-length cDNA encodes a 48 kDa protein, NEMO (NF-κB Essential MOdulator), which contains a putative leucine zipper motif. This protein is absent from 5R cells, is part of the high molecular weight IkB kinase complex, and is required for its formation. In vitro, NEMO can homodimerize and directly interacts with IKK-2. The NEMO cDNA was also able to complement another NF-κB-unresponsive cell line, 1.3E2, in which the protein is also absent, allowing us to demonstrate that this factor is required not only for Tax but also for LPS, PMA, and IL-1 stimulation of NF-kB activity.

Introduction

The Rel/NF- κ B family of transcription factors plays important roles in immune and stress responses, in inflammation, and in apoptosis, regulating the expression of numerous cellular and viral genes (for recent reviews, see Verma et al., 1995; Baldwin, 1996; May and Ghosh, 1998). The NF- κ B activity is composed of homodimers or heterodimers of related proteins that share a conserved DNA-binding and dimerization domain called the Rel homology domain. In most cell types, NF- κ B is sequestered in the cytoplasm bound to inhibitory proteins called I κ B- α , I κ B- β , and I κ B- ϵ . In response to diverse stimuli, including inflammatory cytokines, mitogens, bacterial lipopolysaccharide (LPS), or some viral products,

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active NF-kB is released and translocated to the nucleus as a result of the proteolytic degradation of IkB proteins. Phosphorylation of $I\kappa B\alpha$ on Ser-32 and Ser-36 targets the molecule for degradation by the ubiquitin-26S proteasome pathway. While the processes leading to the degradation of the IkB proteins are relatively well understood, the mechanism by which a variety of distinct signals initiated from the cell membrane are transduced to their common targets, the IkB proteins, remains to be elucidated. A protein kinase activity was identified as a large multisubunit complex that can phosphorylate $I\kappa B\alpha$ at Ser-32 and Ser-36 (Chen et al., 1996; Lee et al., 1997). Most recently, two related kinases have been cloned that contain a catalytic domain at the amino terminus and a leucine zipper (LZ) as well as a helixloop-helix (HLH) motif at the carboxy terminus (Didonato et al., 1997; Mercurio et al., 1997; Regnier et al., 1997; Woronicz et al., 1997; Zandi et al., 1997). Although both of them have been shown to be essential contributors to cytokine-mediated NF-kB activation, understanding of the precise nature of the IkB kinase activity and its regulatory mechanisms awaits further investigation and identification of the other subunits of the kinase complex. Another important issue still unanswered is how discrete activation signals triggered by a variety of known stimulators are integrated to give rise to IkB kinase activity.

One attractive approach to such questions would be the use of somatic cell genetics. Although the diploidy of the mammalian genome presents a major hurdle to a genetic approach, successful establishment of recessive mutants has provided helpful informations on a signaling pathway and a reliable way to identify relevant gene(s) by complementation. Indeed, the Janus kinase family of tyrosine kinases was identified as essential signal transducers for the interferons through a genetic approach (Velazquez et al., 1992; Darnell et al., 1994). Concerning the NF-kB signaling pathways, we have previously reported the characterization of a mutant of the murine pre-B cell line 70Z/3, 1.3E2, which had been isolated by selecting cells unable to express surface IgM following lipopolysaccharide stimulation (Courtois et al., 1997). We have proposed that the 1.3E2 cell line was deficient in a step that is required by several different stimuli to activate NF-kB.

In this report, we present another mutant cell line, 5R, originally isolated as a cellular flat variant of Rat-1 fibroblasts transformed by the Tax protein of human T cell leukemia virus type 1 (HTLV-1). Tax is known to activate transcription from the HTLV-1 long terminal repeat, to cause permanent activation of many cellular transcription factors including NF-kB, and to give rise to cellular transformation (for a review, see Yoshida et al., 1995). 5R cells carry a recessive cellular mutation that abolishes Tax-induced constitutive NF-kB activity, therefore providing a potential means of identifying a critical molecule involved in Tax-mediated NF-кВ activation. Interestingly, 5R cells were found to be resistant to multiple NF-kB activating stimuli besides Tax, suggesting they carried a mutation at a converging regulatory step. We decided to use 5R cells for a genetic

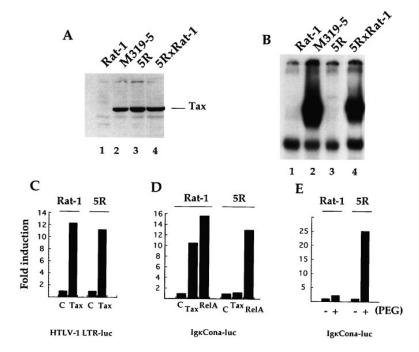


Figure 1. Characterization of 5R Cells

(A) Fifty micrograms of whole-cell extracts derived from wild-type Rat-1 cells (lane 1), the Tax-transformed clone M319-5 (lane 2), the 5R flat revertant (lane 3), and a pool of hybrids between 5R and a Rat-1 derived clone bearing an integrated hygromycin resistance gene (lane 4) were analyzed by immunoblotting using anti-Tax mAb MI73. (B) Five micrograms of nuclear extracts derived from the same cells (as indicated above the lanes) were analyzed by bandshift assay using the κB site derived from the H-2 K^b

the lanes) were analyzed by bandshift assay using the kB site derived from the H-2 Kb promoter as a probe. The NF-kB complex is indicated by a square dot on the right. (C and D) Rat-1 or 5R cells were cotransfected with 0.25 µg of HTLV-1 LTR-luciferase (C) or Igk-luciferase (D) and 1 µg of either empty vector (C) or Tax or relA expression vectors. Luciferase activity was measured after 40 hr. Fold induction over basal level is shown.

(E) Rat-1 or 5R cells (as indicated) were cocultured with Rat-1 cells carrying an integrated \lg_{K} -luciferase plasmid, treated with (+) or without (–) 50% PEG for 1 min, and harvested 12 hr later. Equivalent amount of protein extract was used for the luciferase assay.

complementation approach for the following reasons. First, as the screen we decided to use was based on the NF-κB-dependent expression of a drug resistance gene, the presence of Tax would ensure restoration of a permanent high NF-κB activity following complementation. Second, Rat-1-derived cells grow well in the presence of a high NF-κB activity. Third, 5R cells are expected to show a transformed phenotype following complementation. Here, we describe the genetic complementation of 5R cells by infection with a cDNA expression library cloned into a retroviral vector, demonstrate that expression of the cloned gene, *nemo*, also complements the defect in the 1.3E2 cell line, and show that NEMO is part of the high molecular weight IKK complex and is required for its formation.

Results

Characterization of the Mutant Cell Line 5R

Spontaneous flat revertant cells were isolated from M319-5 cells, a clone of Rat-1 fibroblasts transformed by a mutant Tax protein competent to activate NF-κB but unable to stimulate HTLV-1 long terminal repeat (LTR)-directed transcription (Yamaoka et al., 1996). All of them except one (clone 5R) had lost Tax expression (data not shown). 5R cells express Tax at a level comparable with the parental cells (Figure 1A, lane 3) but are defective in Tax-induced NF-kB DNA binding activity (Figure 1B, lane 3). Stable expression of wild-type Tax failed to retransform 5R cells, while forced expression of constitutively active c-Ha-Ras or v-Src protein transformed 5R cells as efficiently as the parental Rat-1 cells (data not shown). Transient expression of wild-type Tax fully activated HTLV-1 LTR-directed, but not NF-kBdependent, transcription in 5R cells (Figures 1C and 1D). On the other hand, transient expression of RelA or activated c-Ha-Ras strongly stimulated NF-κB- or

serum-responsive element–dependent transcription, respectively, in 5R as well as in Rat-1 cells (Figure 1D and data not shown). These results suggest that 5R cells carry a mutation(s) that abrogates Tax-mediated NF- κ B activation.

We next analyzed the phenotype of the mutation by somatic cell hybridization. Since 5R cells express Tax, they are expected to restore Tax-induced NF- κB activity after hybridization with parental cells if the mutation is recessive. Hybridization of 5R cells with Rat-1 cells carrying an integrated NF- κB -dependent reporter gene induced a strong transcriptional activity when compared with the control hybridization (Figure 1E). We also established a pooled population of stable hybrids between 5R and Rat-1 cells and found that they exhibited a transformed phenotype (data not shown) and contained high NF- κB DNA binding activity (Figure 1B, lane 4). These results indicate that the phenotype of the mutation in 5R cells is recessive and therefore should be amenable to genetic complementation.

Rat-1 cells normally activate NF-kB in response to diverse external stimuli, including tumor necrosis factor α (TNF α), interleukin-1 (IL-1), lipopolysaccharide (LPS), or double-stranded RNA (dsRNA). Interestingly, none of these stimuli was able to induce NF-kB DNA binding activity in 5R cells (Figure 2A). This result was further confirmed by transient transfection with an NF-kBdependent reporter gene (Figure 2B). To identify the step at which NF-κB signaling is affected, we examined the levels of IkB proteins in cells stimulated with LPS. As shown in Figure 2C, LPS stimulation led to a complete loss of $I\kappa B\alpha$ and of $I\kappa B\beta$ in Rat-1 cells followed by reappearance of $I_K B\alpha$ 60 min after stimulation. In contrast, the levels of the two IkB proteins in 5R cells were virtually unaffected by LPS treatment. Taken together, we can conclude that 5R cells carry a recessive mutation(s) at

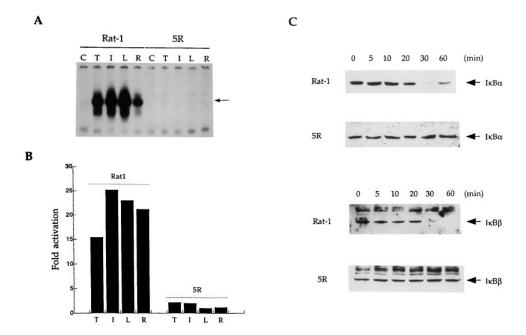


Figure 2. Response of Rat-1 and 5R Cells to NF- κ B Activating Signals (A) Bandshift assay of nuclear extracts from Rat-1 or 5R cells either untreated (none) or stimulated as indicated above the lanes. Stimulation was for 30 min with 10 ng/ml of TNF α , 20 ng/ml of IL-1, 15 μ g/ml of LPS, or 0.1 mg/ml of dsRNA. The arrow marks NF- κ B complex. (B) Transactivation of Ig κ -luciferase transfected Rat-1 or 5R cells by TNF α (T), IL-1 (I), LPS (L), or dsRNA (R). Stimulation was for 3 hr. (C) Immunoblotting analysis of extracts derived from LPS-treated Rat-1 or 5R cells. Cytoplasmic extracts were prepared at the indicated times and 50 μ g analyzed by Western blotting.

a converging regulatory step leading to inducible degradation of I_KB proteins. Finally, we addressed the possibility that 5R cells might be defective in one of the functional I_KB kinases. Stable transfection of 5R cells with plasmids encoding either IKK-1 or IKK-2 did not restore NF-KB activity (data not shown).

Molecular Cloning of NEMO

For complementation experiments, we first established a selection system by preparing sublines of 5R cells capable of expressing an NF-kB-dependent inducible drug resistance gene. A conditional drug resistance gene, ркB2bsrH, contains both a hygromycin resistance gene under the control of the HSV1 thymidine kinase gene promoter and the blasticidin deaminase gene (Izumi et al., 1991) linked to a minimal IL-2 promoter following three repeats of the immunoglobulin κ light chain NF-κBbinding site. Stable transfection of the parental Tax transformed cells with this construct using hygromycin selection followed by selection with blasticidin S resulted in numerous surviving colonies, whereas none could be observed for 5R cells. Hygromycin-resistant 5R clones were tested for survival in the presence of blasticidin S following simple coculture or hybridization with normal Rat-1 cells. One of the 5R clones, h12, was chosen at random for further experiments as being able to survive a high dose of blasticidin S selection after the hybridization but showing absolutely no survival at a low concentration of the drug without the hybridization step. A high NF-kB DNA binding activity was detected in stable h12/Rat-1 hybrids, a result of activation by Tax following complementation of the defect of h12 cells (data not shown).

Approximately 30×10^6 h12 cells were infected with retroviruses carrying a cDNA expression library derived from the T28 murine T cell hybridoma cell line (Whitehead et al., 1995). Viral supernatants were produced by transient transfection of Phoenix cells with the retroviral constructs giving titers in the range of 2 \times 10 5 to 3 \times 105/ml. Selection with blasticidin S was started 36 hr after viral infection. In 20-30 days, a total of more than 40 independent clones was obtained, and 20 were tested for their NF-kB DNA binding activity. All clones except one contained high levels of DNA binding activity and clearly showed a transformed phenotype (Figure 4B, lanes 5-6). Polymerase chain reaction-mediated amplification of genomic DNAs from seven clones resulted in a provirus-derived specific band with a size of 3.2 kb, while 33 other clones carried a 2.8 kb insert. Southern blot analysis of the 3.2 kb insert showed crosshybridization with the 2.8 kb fragment. Sequencing analysis of the amplified 2.8 kb cDNA showed that it contained an open reading frame predicted to encode a previously unknown 48 kDa polypeptide, which we have named NEMO (NF-κB Essential MOdulator) (Figure 3). This molecule is acidic (pl 5.66) and unusually rich in glutamic acid and glutamine (13% each). In addition, it contains a putative leucine zipper motif (amino acids 315-342). To characterize its function, we first transfected Rat-1 or 5R cells with a mammalian expression vector capable of expressing NEMO. Cotransfection of 5R cells with a very small amount of NEMO and an NF-κB-dependent reporter gene resulted in a strong

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MNKHPWKNQLSETVQPSGGPAEDQDMLGEESSLGKPAMLHLPSEQGTPET 50
LQRCLEENQELRDAIRQSNQMLRERCEELLHFQVSQREEKEFLMCKFQEA 100
RKLVERLSLEKLDLRSQREQALKELEQLKKCQQQMAEDKASVKAQVTSLL 150
GELQESQSRLEAATKDRQALEGRIRAVSEQVRQLESEREVLQQQHSVQVD 200
QLRMQNQSVEAALRMERQAASEEKRKLAQLQAAYHQLFQDYDSHIKSSKG 250
MQLEDLRQQLQQAEEALVAKQELIDKLKEEAEQHKIVMETVFVLKAQADI 300
YKADFQAERHAREKLVEKKEYLQEQLEQLQREFNKLKVOCHESARIEDMR 350
KRHVETPQFPLLPAPAHHSFHLALSNQRRSPPEEFPDFCCPKCQYQAPDM 400
DTLQIHVMECI 412

Figure 3. Sequence of the NEMO Protein The putative leucine zipper is boxed.

reporter gene activation by endogenous Tax, whereas its overexpression in Rat-1 cells barely activated the reporter construct (Figure 4A). We then established Rat-1 or 5R cells stably expressing NEMO. As expected, stable expression of NEMO (under the control of the strong CMV promoter) in wild-type Rat-1 cells did not give rise to detectable NF-κB activity (Figure 4B, lane 2). On the other hand, two pooled populations derived from NEMO-transformed 5R cells and two isolated clones showed high levels of NF-κB DNA binding activity

(lanes 7–10), indicating that stable NEMO expression can complement the defect in 5R cells.

A polyclonal antibody was raised against the region encompassing amino acids 60–329 of NEMO and used to analyze its expression in 5R cells. Whereas the protein could be readily detected as a single 48 kDa band in Rat1 cytoplasmic extracts (Figure 4C), no NEMO band could be observed in 5R cells. In addition, we were not able to detect any truncated form of the protein. Thus, the defective phenotype of 5R cells results from the absence of the NEMO protein.

Complementation of the 1.3E2 Mutant Cell Line by NEMO

We have recently reported the characterization of another mutant cell line, the 70Z/3-derived mutant 1.3E2, that exhibits a defect in NF- κ B activation (Courtois et al., 1997). In this cell line NF- κ B is not activated in response to a large set of stimuli, among them LPS, IL-1, PMA, dsRNA, or TNF. This is due to a lack of I κ B α , I κ B β , and I κ B ϵ degradation. Since phosphorylation of I κ B α on Ser-32 and Ser-36 is not observed after stimulation, we proposed that a converging step preceding the I κ B phosphorylation step or the phosphorylation step itself was deficient in 1.3E2.

Since the 1.3E2 phenotype shares many similarities with the 5R phenotype, we tested whether NEMO could

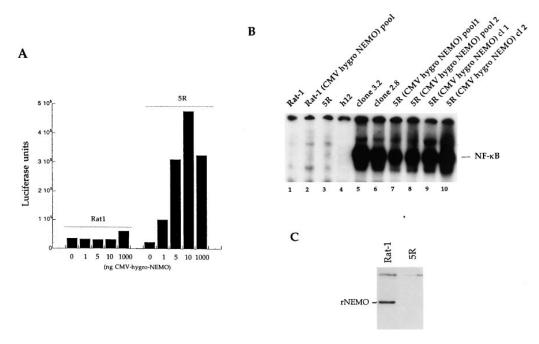


Figure 4. NEMO Complements the Defect in 5R Cells

(A) Rat-1 or 5R cells were transiently transfected with 0.25 μg of $lg\kappa$ -luciferase and the indicated amount of CMV-hygro-NEMO. Luciferase assays were performed as described in Figure 2.

(B) Band shift assay of Rat-1- or 5R-derived cell lines stably expressing NEMO. Five micrograms of nuclear extracts derived from the following cell lines were analyzed as in Figure 1. Lane 1, wild-type Rat-1 cells. Lane 2, a pool of Rat-1 cells transfected with CMV-hygro-NEMO. Lane 3, 5R cells. Lane 4, h12 cells (5R cells containing the inducible blasticidin S resistance gene). Lanes 5 and 6, cDNA library infected h12 clones that survived the blasticidin S selection. The size of the cDNA amplified from each clone is indicated. Lanes 7 and 8, independent pools of 5R cells stably transfected with CMV-hygro-NEMO. Lanes 9 and 10, two representative 5R cell clones obtained by stable transfection with CMV-hygro-NEMO.

(C) Immunoblotting analysis of cytoplasmic extracts (100 μg) derived from Rat-1 or 5R cells was carried out with an antibody specific for NEMO, rNEMO, rat NEMO.

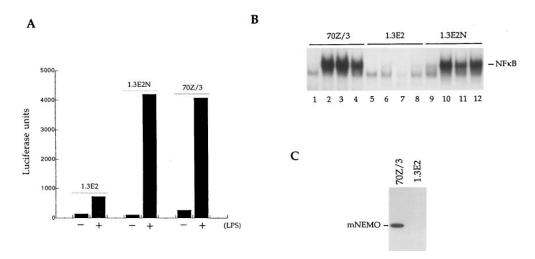


Figure 5. NEMO Complements the Defect in 1.3E2 Cells

(A) 1.3E2, 1.3E2 stably transfected with NEMO (1.3E2N), and 70Z/3 cells were transiently cotransfected with 3 μ g of Ig κ -luciferase and 6 μ g of CMV-hygro-NEMO. After 24 hr, cells were split in two and left untreated (–) or stimulated (+) with 15 μ g/ml LPS. Luciferase assays were performed as described in Figure 2.

(B) Bandshift assay of complemented 1.3E2 cells. 70Z/3 (lanes 1–4), 1.3E2 (lanes 5–8), or a pool of 1.3E2 cells stably transfected with CMV-hygro-NEMO (1.3E2N, lanes 9–12) were left untreated (lanes 1, 5, and 9) or stimulated with 15 μ g/ml LPS (lanes 2, 6, and 10), 100 ng/ml PMA (lanes 3, 7, and 11), or 20 ng/ml IL-1 (lanes 4, 8, and 12). Five micrograms of nuclear extracts were then analyzed by bandshift using the H-2 K^b derived κ B site.

(C) Immunoblotting analysis of cytoplasmic extracts (100 μ g) derived from 70Z/3 or 1.3E2 cells was carried out with the NEMO antiserum. mNEMO, mouse NEMO.

complement 1.3E2. Strikingly, as shown in Figure 5A, transient transfection of 1.3E2 with a vector expressing NEMO allowed the recovery of a wild-type NF- κ B activation level after LPS stimulation. Such an effect was clearly stimulus-specific, indicating that NEMO overexpression by itself was unable to activate NF- κ B. Complemention was also observed in the case of two other stimuli, IL-1 and PMA, although with less efficiency in the latter case (data not shown).

1.3E2 cells stably expressing NEMO (1.3E2N) were also prepared and tested for complementation. A mobility shift experiment presented in Figure 5B confirmed the results of the transient transfection experiments described above. NF- κ B activation in response to LPS, IL-1, or PMA was found to be similar in wild-type 70Z/3 and 1.3E2N. Moreover, an immunoblot analysis revealed that NEMO is undetectable in 1.3E2 cells (Figure 5C). These results demonstrate that, as for 5R cells, the phenotype of the 1.3E2 mutant cell line is due to the absence of NEMO.

NEMO Is Part of the IκB Kinase Complex

Since NEMO appears to be critically involved in NF- κ B activation by a large set of stimuli and complements cells defective in I κ B phosphorylation, an attractive possibility would be that it constitutes a subunit of the 600–800 kDa kinase complex that phosphorylates I κ B. Therefore, we investigated whether NEMO is associated with the inducible I κ B kinase activity (Figure 6). To demonstrate this point, we carried out immune complex kinase assays on Rat-1 or 5R cells. The antiserum against NEMO immunoprecipitated a specific endogenous I κ B α kinase activity from wild-type cells stimulated with

TNF α . Absence of kinase activity in NEMO immunoprecipitates from 5R cells and lack of phosphorylation of a mutant I κ B α polypeptide (S32A, S36A) established the specificity of the antiserum and kinase activity, respectively. Thus, NEMO is associated with an inducible endogenous I κ B α kinase activity. As reported previously, an anti-IKK-1 antibody brought down a specific I κ B α kinase activity from wild-type cells stimulated with TNF α for 5 min. Interestingly, no inducible I κ B α kinase activity was observed in IKK-1 precipitates from 5R cell extracts.

To confirm that NEMO is an integral part of the $I\kappa B$ kinase complex and to determine whether it is stably associated with it before stimulation, S100 extracts were prepared from Rat-1 cells and fractionated on a Superose 6 gel filtration column. Elution of the $I\kappa B$ kinase, monitored with an anti-IKK-1 antibody, was mostly observed in fractions containing proteins of 600–800 kDa, as previously reported (Figure 7A). When we looked for NEMO elution, an identical profile was obtained. Immunoprecitation of the NEMO-containing fractions with an anti-NEMO antibody allowed us to coimmunoprecipitate IKK-1 (Figure 7B). NEMO is therefore a stable component of the 600–800 kDa $I\kappa B$ kinase complex.

Quite remarkably, when 5R extracts were analyzed with the IKK-1 antibody, the elution peak appeared shifted toward fractions containing proteins of 300–450 kDa instead of 600–800 kDa (Figure 7A). Since the overall elution profile, as checked either by silver staining (Figure 7A, top panel) or by Western blotting against ReIA (Figure 7A, bottom panel) or p105 (data not shown), was identical between Rat-1 and 5R, this observation demonstrated the requirement of NEMO for building a high molecular weight IkB kinase complex. Moreover,

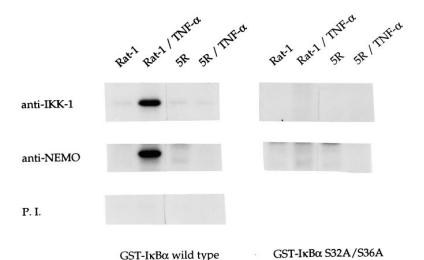


Figure 6. NEMO Is Associated with an Inducible Endogenous $I_KB\alpha$ Kinase Activity Rat-1 or 5R cells were treated for 5 min with or without TNF α (10 ng/ml). Cytoplasmic extracts were immunoprecipitated with either preimmune serum (P.I.), anti-IKK-1 antibody (anti-IKK-1), or NEMO antiserum (anti-NEMO), and specific $I_KB\alpha$ kinase activity was determined by an in vitro immune complex kinase assay with GST- $I_KB\alpha$ (1–72) wild-type or GST- $I_KB\alpha$ (1–72) S32A/S36A mutant protein as substrates.

the absence of I_KB kinase activity in 5R cells after stimulation (see above) indicates that the lower molecular weight kinase complex is refractory to activation.

NEMO Can Form Homodimers and Interacts Directly with IKK-2

The presence of a leucine zipper-like motif in NEMO led us to ask whether this molecule could dimerize. Glutaraldehyde cross-linking experiments (Figure 7C) demonstrated that NEMO was indeed able to form homodimers. The possible role of the leucine zipper-like region in this dimerization is currently under investigation.

Since NEMO is part of the IkB kinase complex, we also looked for direct interactions with known components of the complex, namely the two catalytic subunits IKK-1 and IKK-2. We carried out an in vitro analysis using 35S-labeled proteins translated in wheat germ extracts (WGE). After cotranslation of VSV-IKK-2 and NEMO followed by anti-VSV imunoprecipitation, we readily detected NEMO in the immunoprecipitate (Figure 7D). The converse experiment, using NEMO plus VSV-IKK-2 and immunoprecipitating with anti-NEMO allowed the detection of VSV-IKK-2 in the immunoprecipitate (data not shown). Interestingly, such an interaction could barely be observed with IKK-1, suggesting a potential functional divergence between the two IKKs (data not shown).

Discussion

The recent description of a high molecular weight cytoplasmic complex able to phosphorylate $I_KB\alpha$ on Ser-32 and Ser-36 (Chen et al., 1996; Lee et al., 1997) has prompted intense studies, which culminated a few months ago with the cloning of two kinases, named IKK-1 and IKK-2, or IKK α and IKK β (Didonato et al., 1997; Mercurio et al., 1997; Regnier et al., 1997; Woronicz et al., 1997; Zandi et al., 1997). Two approaches were used to this end: one involved biochemical purification from a cytoplasmic extract derived from TNF-treated HeLa cells (Didonato et al., 1997; Mercurio et al., 1997; Zandi et al., 1997), while the other used a two-hybrid screen

using as a bait NIK, a protein kinase previously shown to be involved in TNF- and IL-1-induced NF-кB activation (Regnier et al., 1997; Woronicz et al., 1997). The cloned kinases were postulated to directly phosphorylate Ser-32 and Ser-36 of $I\kappa B\alpha$, although this has not been formally demonstrated. The reason for this uncertainty is that all kinase assays reported so far rely on immunoprecipitation of transfected or in vitro translated IKK, therefore leaving open the possibility that the "true" IkB kinase is coprecipitated together with IKK and the rest of the high molecular weight complex. Immunoprecipitation of one kinase from extracts of cells transfected with the two kinases results in the coprecipitation of the second kinase, and a more detailed study has demonstrated that heteroassociation was favored over homoassociation. The sequence of IKK-1 and IKK-2 has revealed two interesting features: a leucine zipper and a HLH motif. Deletion of the LZ in one of the kinases results in the abrogation of coimmunoprecipitation with either itself or the other kinase and a strong reduction in the resulting kinase activity. However, it is unclear whether the LZ motif is required for direct interaction between the kinase subunits or between the kinase(s) and some other component of the complex. Deletion of the HLH motif leaves the coimmunoprecipitation of the two kinases intact, but it strongly reduces the resulting kinase activity. In the assays used in the above mentioned papers, transfected IKK-2 seems to exhibit a stronger basal kinase activity when compared to IKK-1 (Mercurio et al., 1997; Zandi et al., 1997). Zandi et al. (1997) also observed that cotranslation of the two kinases in wheat germ extracts resulted in no IkB kinase activity, suggesting that either posttranslational modifications or additional components of the complex (or both) are required. We also observed that cotranslation of the two kinases in wheat germ extracts precluded their association (S. T. W., unpublished data). One possibility is that the kinase subunits need to be incorporated into the 600-800 kDa complex in order to be fully active and that some critical components of the complex are absent in wheat germ extracts. In any case, all these data emphasize the importance of identifying additional components of the complex.

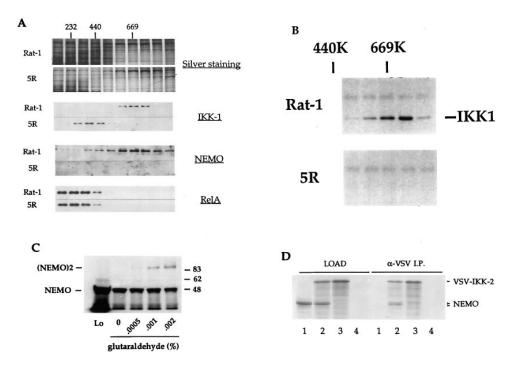


Figure 7. NEMO Is a Subunit of the IkB Kinase Complex

(A) Gel filtration analysis of NEMO and IκB kinase complex in Rat-1 and 5R cells. S100 extracts were prepared as described in Experimental Procedures and fractionated through a Superose 6 column. Fractions were analyzed by Western blotting, using antibodies specific for IKK-1 or NEMO. Analysis of NF-κB/IκB elution, using an anti-relA antibody, is also shown. To demonstrate identical elution of Rat-1 and 5R extracts, the protein profile from each fraction was analyzed by silver staining (upper panel).

(B) Coimmunoprecipitation of IKK-1 with NEMO. Positive NEMO fractions from Rat-1 and the equivalent fractions from 5R cells were imunoprecipitated with anti-NEMO, run through a 7.5% SDS-Laemmli gel, and immunoblotted with anti-IKK-1.

(C) NEMO forms homodimers. The NEMO protein was in vitro synthesized in wheat germ extract and treated with the indicated concentrations of glutaraldehyde. The reactions were immunoprecipitated with NEMO antiserum and analyzed on a 8% SDS-polyacrylamide gel. The positions of the NEMO monomer and NEMO dimer ([NEMO]₂) are indicated. Lo, in vitro translated product.

(D) In vitro interaction between NEMO and IKK-2. Untagged NEMO (lane 1), VSV-IKK-2 (lane 3), or both molecules (lane 2) were in vitro translated in wheat germ extract (Load). The 35-labeled products were then precipitated with anti-VSV antibody (VSV-IP). Lane 4 represents unprogrammed wheat germ extract. The relevant proteins are indicated on the right.

One approach aimed at identifying components of the NF-kB signaling pathway that has not been widely used so far is to generate mutant cell lines which are unresponsive to one or several NF-kB activating signals and to try and complement these cell lines with genomic or cDNA libraries (Ting et al., 1996). We have used here a spontaneous mutant (called 5R) of a HTLV-1 transformed Rat-1 fibroblastic cell line, which had lost its transformed morphology. This mutation was accompanied by disappearance of Tax-induced NF-κB activity, as measured by bandshift and transactivation assays. In addition, LPS-, IL-1-, dsRNA-, or TNF-induced NFкВ DNA binding activity could not be observed in the 5R cell line. However, other signaling pathways seemed to be still functional. Importantly, cell fusion experiments demonstrated that the mutation was recessive. All these observations prompted us to try to complement this cell line. The selection was based on introduction into these cells, prior to complementation, of a gene encoding resistance to the antibiotic blasticidin S driven by multimerized NF-κB-binding sites. Only the complemented cells would be expected to become resistant to blasticidin S treatment, a result of transactivation of the blasticidin S resistance gene by endogenous Tax. More than 40 independent blasticidin S-resistant clones were isolated, and bandshift analysis demonstrated the presence of a p50/relA complex in 19 analyzed clones out of 20, with an intensity similar to that observed following stimulation of wild-type Rat-1 cells with LPS or TNF. PCR amplification of DNA from 40 independent clones using primers localized in the flanking regions of the retroviral vector yielded two cross-hybridizing fragments of 2.8 and 3.2 kb. Sequencing of the amplified cDNA revealed that the 2.8 kb insert contains an open reading frame encoding a previously undescribed 412 amino acid protein, which we call NEMO. This protein is acidic (pl 5.66), unusually rich in glutamic acid and glutamine (13% each), and also contains a putative leucine zipper motif (amino acids 315–342).

Transfection of NEMO complemented the mutation in 5R cells. This led us to conclude that NEMO is necessary for activation of NF- κ B by Tax. However, the presence of endogenous Tax in the 5R cell line precluded the analysis of NEMO involvement in other NF- κ B activation pathways. This problem was circumvented by the use of 1.3E2, another mutant cell line that we previously characterized (Courtois et al., 1997). NF- κ B activation, degradation of the three known 1κ B inhibitors, as well as

induced phosphorylation of $I_{\kappa}B_{\alpha}$ could not be observed following PMA, LPS, IL-1, or dsRNA treatment of this cell line. We stably introduced the NEMO cDNA into 1.3E2 and observed that NF- κ B activation by at least three of these stimuli (LPS, PMA, and IL-1) was restored. Therefore the NEMO protein is involved in the response to at least four NF- κ B activating stimuli.

An interesting conclusion we can draw from complementation of the 5R cells, which regain a transformed phenotype when stably transfected with NEMO, is that NF- κ B activity seems to be required for cell transformation by Tax (at least in this cell system). There have been conflicting data in the literature concerning the actual involvement of NF- κ B in Tax-induced transformation (Smith and Greene, 1991; Kitajima et al., 1992; Yamaoka et al., 1996), and the formal possibility exists that NEMO is involved in another signaling pathway, beside that of NF- κ B, which would be required for transformation. Clearly more work is needed to unambiguously answer this question.

The next question concerned the actual function of NEMO. Since this molecule appears to be involved in all tested NF- κ B activating pathways, an obvious possibility was that it constituted one subunit of the high molecular weight I κ B kinase complex. We obtained three types of arguments in favor of this hypothesis. First, immunoprecipitation of NEMO from Rat-1 cells pulled down a bona fide I κ B α kinase activity, specific for the two N-terminal serines. Second, NEMO elutes as a 600–800 kDa peak from a gel filtration column performed on extracts from unstimulated Rat-1 cells, as does IKK-1. Third, immunoprecipitation of NEMO from Rat-1 fractions ranging from 600–800 kDa brings down IKK-1.

We then tested the possible interaction of NEMO with the two catalytic subunits of the complex, IKK-1 and IKK-2. In vitro cotranslation of IKK-2 and NEMO in wheat germ extract followed by immunoprecipitation demonstrated that the two proteins could interact with each other. In contrast, an interaction between NEMO and IKK-1 could barely be detected under these conditions. We also demonstrated that NEMO can form homodimers.

The fact that NEMO interacts with IKK-2 and apparently not with IKK-1 introduces an asymmetry between the two kinases. The respective functions of these two molecules, however, are still unclear; in particular, the question of whether the two serines in the N-terminal regions of the three inhibitors are phosphorylated by the same or different kinases is currently unknown. Similarly, the three inhibitors might be phosphorylated by the same complexes or by different ones. The issue of the actual function of NEMO in the complexes will be addressed by a detailed molecular analysis of the IkB kinase complex in 5R and 1.3E2 cells, as well as by a mutational analysis of NEMO followed by reintroduction of the mutated molecules into 5R or 1.3E2 cells. These points are currently under investigation.

Another intriguing question concerns the actual defects in 5R and 1.3E2 cells. Immunoblot analysis indicates that the NEMO protein is absent from both 5R and 1.3E2 cells and that in 5R cells, which exhibit no IKK1-associated kinase activity, the high molecular weight

complex does not seem to exist. Although unlikely, the formal possibility exists that a complex which would not contain IKK-1 exists in these cells, but in any case they exhibit no inducible phosphorylation of IkB. Interestingly, IKK-1 can be detected in a 300–450 kDa complex in 5R cells, therefore indicating that NEMO is required for the formation of a 600–800 kDa functional IKK complex, and probably plays a role as a structural component of this complex. Further work will be needed to determine which components of the functional complex (besides NEMO) are missing from this smaller nonfunctional complex and which components of the complex (besides IKK-2) directly interact with NEMO.

It was unexpected that two independently isolated mutant cell lines could be complemented by the same cDNA. The selection for LPS-unresponsive derivatives of 70Z/3 yielded several types of mutant cell lines, but only 1.3E2 was also unresponsive to other NF-κB activating stimuli, and the fact that it grows faster than the wild-type 70Z/3 probably facilitated its isolation. In Taxtransformed Rat-1 cells, 5R was the only NF-kB-defective cellular revertant that could be isolated. This leads to the intriguing possibility that mutating the nemo gene might be the only means of knocking out NF-kB activation by a single gene mutation. Future inactivation experiments of the other components of the complex (including IKK-1 and IKK-2) will tell whether this hypothesis is correct and whether NEMO is a relevant target for future drugs aimed at blocking NF-kB activation.

Experimental Procedures

Cells and Transfections

The 70Z/3 murine pre-B cell line and the NF-κB unresponsive mutant 1.3E2 were maintained in RPMI medium supplemented with 10% fetal calf serum and 50 μ M β -mercaptoethanol. 70Z/3 and 1.3E2 cells were transiently transfected as described (Courtois et al., 1997). Isolated stable clones were prepared as described (Whiteside et al., 1995). Rat-1 and 5R cells were grown in DMEM supplemented with 10% fetal calf serum and transfected using the calcium phosphate coprecipitation method. For measurement of luciferase activity in transiently transfected Rat-1 or 5R cells, approximately 2 imes105 cells were transfected with 0.25 μg of a reporter plasmid, 0.25 μg of EF1-lacZ plasmid, and 1 μg of either vector or effector plasmid. Cells were harvested 40-45 hr after transfection. The amount of lysate used for luciferase assay was determined on the basis of β -galactosidase activity. The results shown are representative of one experiment carried out in duplicate and averaged. Each experiment was repeated at least three times, with similar results.

Phoenix-Éco packaging cells were a kind gift of G. Nolan (Stanford University).

Plasmids

A BLAST search of GenBank with the human IKK-1 cDNA sequence revealed the presence of an EST clones encoding for a single, IKK-1-related cDNA. This clone was obtained from the UK HGMP, and the cDNA insert was used to screen an adult human liver cDNA library. Positively hybridizing phage were isolated, and both strands of the largest insert obtained were sequenced by the dideoxy termination method (Sequenase, USB). IKK-2 coding sequences were amplified by PCR and inserted into vectors that allowed the in vitro and in vivo expression of proteins fused to the VSV epitope. Rat IKK-1 was amplified by PCR from an EST clone and subcloned into the same vector. The plasmids Igk-luciferase and SRE-luciferase have been described previously (Courtois et al., 1997); HTLV-1 LTR-luciferase was a kind gift of P. Jalinot (Ecole Normale Supérieure de Lyon).

The plasmid Igk2bsrH was constructed by ligating a 1.5 kb HindIII/

BamHI fragment of the plasmid pSV2bsr (Izumi et al., 1991) with a 5.1 kb HindIII/BamHI fragment of the plasmid cx12lacZ- κ B (Fiering et al., 1990), which contains three tandem copies of the NF- κ B oligonucleotide derived from the Ig κ sequence (TCAGAGGGACTT TCCGAG) followed by a minimal IL-2 promoter.

A Tax expression vector, pCntax, was constructed by inserting a BamHI fragment of the plasmid pUCwtax (Yamaoka et al., 1996) containing the entire coding sequence of Tax to the unique BamHI site of pCMV-Neo-Bam vector (Baker et al., 1990).

A 2.8 kb PCR product derived from genomic DNA of a blasticidin S-resistant 5R clone was obtained using primers located in the retroviral vector pCTV1 (Whitehead et al., 1995). This PCR product was then digested with Sall, subcloned into pBluescript for sequencing, or into the Xhol site of the CMV-hygro vector (a kind gift of F. Aurade, Institut Pasteur). Full construction details are available on request.

Reagents

LPS, PMA, poly (I-C), chloroquine, and polybrene were from Sigma. Recombinant hIL-1 β was from Biogen (Geneva, Switzerland). Recombinant TNF α was from Genzyme. Blasticidin S was purchased from ICN. Absence of endotoxin contamination in all these reagents, except LPS, was checked with a polymyxin B assay (Shapiro and Dinarello, 1995).

Antisera

Rabbit antiserum against $I\kappa B\alpha$ was a kind gift of J. DiDonato and M. Karin (UCSD). Anti-VSV was mouse monoclonal P5D4. Anti-Tax was mouse monoclonal M173 (Mori et al., 1987). Anti-IKK-1 antibody was from Santa Cruz. Anti NEMO rabbit polyclonal antiserum (serum 44106) was raised against a TrpE fusion of a fragment encompassing amino acids 30–329 of murine NEMO in the Path11 vector (Spindler et al., 1984).

Preparation of Cell Extracts

Cells were washed with PBS and resuspended at 10 6 cells/10 μ l in hypotonic solution (10 mM HEPES [pH 7.8], 10 mM KCl, 2 mM MgCl $_2$, 1 mM DTT, 0.1 mM EDTA supplemented with a protease inhibitor cocktail [Boehringer]). After 10 min at 4 $^\circ$ C, NP40 was added to 1% and the cells centrifuged in a microfuge for 20 s. The supernatant, containing the cytoplasmic fraction, was recovered. One volume of 2× Laemmli buffer containing 20% β -mercaptoethanol was added, and the sample was boiled for 5 min. The nuclear pellet was briefly washed with hypotonic buffer and resuspended in 40 μ l of extraction buffer (50 mM HEPES [pH 7.8], 50 mM KCl, 350 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, 10% glycerol). After a 30 min incubation on ice, with occasional agitation, the DNA was pelleted by centrifuging at 14000 rpm for 10 min. The supernatant, containing the nuclear fraction, was recovered and quickly frozen on dry ice. Samples were stored at -80° C.

Preparation of S100 Extracts and Gel Filtration Analysis

Fifty million cells were washed in PBS and resuspended in 500 μl of 50 mM Tris (pH 7.5), 1 mM EGTA. Cells were lysed by thirty passages through a 26-gauge needle. After centrifugation for 10 min at 15000 rpm, the supernatant was recovered and complemented with 1 mM DTT, 0.025% Brij 35, and a cocktail of proteases and phosphatases inhibitors. S100 were prepared by centrifuging the cytoplasmic extracts for 30 min at 52000 rpm in a TLA 100.2 rotor (Beckman). After adding 10% glycerol, the S100 extracts were quickly frozen in dry ice and stored in liquid nitrogen. Gel filtration chromatography was carried out on a Superose 6 column (Pharmacia) precalibrated with aldolase (158 kDa), catalase (232 kDa), ferritin (440 kDa), and thyroglobulin (669 kDa). Five hundred microliter fractions were recovered and directly analyzed by Western blotting or immunoprecipitated with anti-NEMO. Silver staining of the fractions was performed with a Silver Stain Plus Kit (Biorad).

Western Blot Analysis

Proteins from cytoplasmic extracts were fractionated on 10% SDS-polyacrylamide gels, transferred onto Immobilon membranes (Millipore), and blots were revealed with an enhanced chemiluminescence detection system (ECL, Amersham).

In Vitro Translation and Cross-Linking

Translations and coimmunoprecipitation experiments were performed as described previously using TNT kits (Promega) (Kieran et al., 1990). For dimerization experiments, translation reactions were diluted thirty times with phosphate buffered saline, treated with glutaraldehyde at room temperature for 20 min, with 100 mM of Tris-HCI (pH 7.4) for 20 min, and subjected to immunoprecipitation after addition of an equal volume of TNT buffer (NaCl 200 mM, Tris-HCI 20 mM [pH 7.5], Triton X-100 1% supplemented with protease and phosphatase inhibitors).

Immunoprecipitations and Kinase Assays

Cytoplasmic extracts were subjected to immunoprecipitation with anti-IKK-1 antibody, anti-NEMO, or preimmune serum in TNT buffer and collected on protein A-Sepharose beads, which were then washed three times with TNT buffer and three times with kinase buffer (20 mM HEPES, 10 mM MgCl $_2$, 100 μ M Na $_3$ VO $_4$, 20 mM β -glycerophosphate, 2 mM DTT, 50 mM NaCl [pH 7.5]). Kinase reactions were for 30 min at 30°C using 5 μ Ci of [γ - 3 P]-ATP and GST-I $_{\rm K}$ B $_{\rm C}$ (1–72) wild-type or GST-I $_{\rm K}$ B $_{\rm C}$ (1–72) S32A/S36A mutant protein as substrates. The reaction products were analyzed on 10% SDS-polyacrylamide gels and revealed by autoradiography for 3 hr at room temperature.

Electrophoretic Mobility Shift Assays

Five micrograms of nuclear extracts were added to 15 μ l of binding buffer (10 mM HEPES [pH 7.8], 100 mM NaCl, 1 mM EDTA, 10% glycerol final), 1 μ g poly (dl-dC), and 0.5 ng 32 P-labeled κ B probe derived from the H-2Kb promoter (Kieran et al., 1990), and incubated for 30 min at room temperature. Samples were run on a 5% polyacrylamide gel in 0.5 \times TBE.

Viral Stocks and Infection

T28 cells, a murine T cell hybridoma (Pyszniak et al., 1994), were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Total mRNA from exponentially growing T28 cells was used as template for cDNA synthesis, using random hexamer primers. Procedures for cDNA synthesis and cloning were as described previously (Whitehead et al., 1995). The cDNA was ligated into pCTV1 (Whitehead et al., 1995), yielding 3.5×10^6 cDNA clones. Complexities of the libraries were as follows: L35, 470,000 clones (3.5 kb and up); L36, 600,000 clones (2.2-3.5 kb). The Phoenix-Eco packaging cell line was used for transient transfection with DNA from the L35 or L36 libraries. To determine the virus titer on 5R cells, a cDNA library (L20) cloned into the pCTV3 vector (Whitehead et al., 1995), which carries a hygromycin resistance gene, was transfected by the calcium phosphate method into Phoenix-Eco cells, and the resultant supernatants were titered by the appearance of hygromycin resistant 5R cells. This library produced viral titers of \sim 2-3 \times 10 5 /ml. We produced viral supernatants for complementation experiments by transfecting approximately 1.5 \times $10^7\,Phoenix$ cells plated 24 hr before with 20 μg of the L35 or L36 library DNA in the presence of 25 μM chloroquine. Supernatants were recovered every 12 hr from 36-72 hr after transfection and either immediately used for infection of h12 cells or snap frozen in dry ice and stored at -80°C. Approximately 106 h12 cells were plated 12-15 hr before infection on a 100 mm petri dish and exposed to 3 ml of viral supernatant in the presence of 3 ml of conditioned medium and 10 µg/ml of polybrene. Twelve hours after starting the infection, the viral supernatant was removed and cells were cultured for an additional 24 hr in normal growth medium. Blasticidin S was added to a final concentration of 10 $\mu g/ml$ 36 hr after infection. The selection medium was replaced at least every 5 days, and the resultant cell clones were isolated with cloning cylinders. We used a total of $30\,\times\,10^6\,h12$ cells for infection with virus stock obtained using 20 ug of L35 or L36 library DNA and finally isolated a similar number of independent cell clones for the two cDNA libraries.

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GenBank Accession Number

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