Phorbol Esters and Cytokines Regulate the Expression of the NEMO-related Protein, a Molecule Involved in a NF-κB-independent Pathway*

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The NF-κB signaling pathway plays a crucial role in the immune, inflammatory, and apoptotic responses. Recently, we identified the NF-κB Essential Modulator (NEMO) as an essential component of this pathway. NEMO is a structural and regulatory subunit of the high molecular kinase complex (IKK) responsible for the phosphorylation of NF-κB inhibitors. Data base searching led to the isolation of a cDNA encoding a protein we called NRP (NEMO-related protein), which shows a strong homology to NEMO. Here we show that NRP is present in a novel high molecular weight complex, that contains none of the known members of the IKK complex. Consistently, we could not observe any effect of NRP on NF-κB signaling. Nonetheless, we could demonstrate that treatment with phorbol esters induces NRP phosphorylation and decreases its half-life. This phosphorylation event could only be inhibited by K-252a and staurosporin. We also show that de novo expression of NRP can be induced by interferon and tumor necrosis factor α and that these two stimuli have a synergistic effect on NRP expression. In addition, we observed that endogenous NRP is associated with the Golgi apparatus. Analogous to NEMO, we find that NRP is associated in a complex with two kinases, suggesting that NRP could play a similar role in another signaling pathway.

The transcription factor NF-κB plays a pivotal role in many cellular processes such as immune responses, inflammation, and apoptosis (1, 2). NF-κB is composed of homo- and heterodimers of various members of the NF-κB/Rel family (3, 4) and is retained in an inactive form in the cytoplasm by an inhibitory protein belonging to the IκB family, mainly represented by IκBα, IκBβ, and IκBε (5–7).

In response to diverse stimuli, including IL-1Ã, LPS, TNFα, or PMA, as well as several viral proteins, active NF-κB translocates to the nucleus as a result of the complete proteolytic degradation of the IκB proteins. This mechanism has been best studied for the inhibitor IκBα and demonstrated to involve phosphorylation on two specific serine residues (8–13) followed by polyubiquitination and degradation by the 26S proteasome (14). More recently a specific serine protein kinase activity responsible for IκBα phosphorylation has been identified as a large cytoplasmic complex (600–800 kDa) containing two catalytic subunits (IKKIα and IKKβ) (15–19). IKKa and IKKβ are related molecules of 85 and 87 kDa, respectively, and share 50% sequence similarity. Both proteins contain NH2-terminal kinase domains, leucine zipper, and helix-loop-helix motifs (16, 19). In vitro phosphorylation studies have shown that both kinases can phosphorylate IκBα on serines 32 and 36, but IKKβ is more active in this regard.

Recently, we have cloned, by complementation of an NF-κB unresponsive cell line, a third component of the IKK complex (20), that we called NEMO (NF-κB Essential Modulator). NEMO is a 48-kDa glutamine-rich protein, which lacks a catalytic subunit, but contains two coiled-coil motifs, a leucine zipper, and a COOH-terminal zinc finger (20). NEMO is a regulatory and structural subunit of the complex which seems to interact directly with IKKβ, but not with IKKa (20). Studies with NH2-terminal deletion mutants of NEMO show that the first 235 residues contain the site of interaction with IKKβ (21). The human homolog of NEMO, IKKγ1/IKKAP, has been cloned after purification of the IKK complex (21, 22).

Data base searching led to the isolation of a cDNA encoding a NEMO-related protein (NRP), which shares 53% sequence similarity with NEMO. NEMO and NRP were also identified in a yeast two-hybrid screen using an adenovirus protein (Ad E3-14.7K) as a bait. Interestingly, these proteins, named FIP-3 and FIP-2, respectively, could block the anti-apoptotic activity of the E3-14.7K protein after TNFα stimulation (23, 24).

In this study, we have characterized NRP more thoroughly. In order to determine whether NRP is involved in NF-κB signaling, we investigated whether it is associated with the IKK complex, or whether it could complement a NEMO-deficient cell line. We could not observe any role of NRP in NF-κB signaling, but we found that the COOH-terminal zinc finger of NRP can functionally replace that of NEMO. We could demonstrate that NRP is present in a high molecular weight complex, smaller than the IKK complex, and is associated with two kinase activities. These results demonstrate that NRP could fulfill a similar regulatory function to that of NEMO in a non-NF-κB-dependent pathway. Moreover in an effort to characterize the signaling pathways that might target NRP, we...
showed that PMA stimulation induces its phosphorylation. This phosphorylation was inhibited by K-252a and staurosporin. Finally, we also demonstrated that NRP expression is synergistically induced by interferon and TNFα.

**MATERIALS AND METHODS**

**Cells**

70Z/3 is a murine pre-B cell line and its variant 1.3E2 is a NEMO-deficient cell line (20). E29.1 is a CD4-negative, CD8-negative mouse T-cell hybridoma (kindly provided by P. Truffa Bachi). Jurkat is a human T-cell cell line. These cells were maintained in RPMI medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C in 5% CO2. In the case of 70Z/3 and 1.3E2, 50 μM β-mercaptoethanol were added in the medium. HeLa cells and 293T cells were maintained in high glucose Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C in 7% CO2.

**Antisera**

The antisera used were the following: anti-IKK2 (Santa Cruz, ref. H470) is a polyclonal rabbit antisera. Anti-CD28.2 is a murine monoclonal antibody directed against human CD28 (Immunotech). T cells were activated by stimulation with an anti-TCR murine monoclonal antibody (Vit-3) (kindly provided by W. Knapp). Anti-NEMO rabbit polyclonal antibody (serum 44106) was raised against a TrpE fusion protein encompassing amino acids 30–329 of murine NEMO (20). Anti-NRP (serum 46096) is a polyclonal rabbit antisera generated against a TrpE fusion protein encompassing amino acids 84–164 of human NRP.

**Plasmids**

NRP open reading frames were amplified by the polymerase chain reaction. Expression vectors for transfection into 1.3E2 cells were obtained by subcloning cDNAs encoding NEMO, NRP, or their derivatives into the plasmid pCDNA-3 (Invitrogen). NEMO ΔZF represents amino acids 1–385 of NEMO. For the cloning of NEMO ΔZF-NRP ZF, NRP plasmid was digested with BstEII/EcoRI and this fragment was ligated into the XhoI site of the NEMO plasmid. Details of all the constructions used in this article are available upon request.

**Total Extracts of Treated and Non-treated Cells**

Treatment with Chemicals—Cells (5 × 10⁶) were incubated for the indicated time at 37 °C in 1 ml of regular growth medium containing 100 IU/ml TNFα (Pharmingen), 20 ng/ml IL-1, 10 ng/ml interferon α/β, 10 ng/ml interferon γ, 15 μg/ml LPS (Sigma), 50 ng/ml NMA (Sigma), 1 μM calcium ionophore (Sigma), or 50 μg/ml cycloheximide (Sigma). Treatment of the cells with kinase inhibitors or activators was performed at 37 °C for 20 min and was followed by PMA stimulation for 30 min.

**Secondary Structure Prediction Analysis**

This analysis was performed using the ExPASy (Expert Protein Analysis System) proteomics server of the Swiss Institute of Bioinformatics (SIB).

**Immunoprecipitations**

Cells were lysed as described above. Specific polypeptides were then recovered by immunoprecipitation from equivalent amounts of cellular proteins (1 mg). Immune complexes were collected with Staphylococcus aureus protein A (Pansorbin, Calbiochem). After washing the immunoprecipitates 3 times in lysis buffer, the proteins were resolved by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. Subsequent immunoblots were obtained using the protocol outlined below.

**Immunoblots**

Immunoblots were performed according to a previously described protocol (25). We used antisera, biotinylated protein A (Interchim Pierce), and streptavidin-horseradish peroxidase (Amersham Pharmacia Biotech) at 1/1000 dilution. Proteins transferred to Immobilon membranes (Millipore) were revealed with the SuperSignal chemiluminescent substrate (Substrate) and immunoreactive products were detected by autoradiography.

**Immunofluorescence**

Cells were lysed with CHIRIS buffer as described above. 1 mg of proteins from the lysates were incubated with 5 μl of the NEMO or NRP antibody. The immunoprecipitations were performed as described above, except that the immunoprecipitates were washed 3 times with 1 × CHIRIS followed by 3 washes with a kinase buffer containing: 10 μM Hapes, pH 7.5, 10 mM MgCl2, 100 μM Na3VO4, 20 μM β-glycerophosphate, 10 μM DTT, 50 mM NaCl. The kinase reactions were conducted as described (20) for 30 min at 30 °C in the kinase buffer, in the presence of 12.5 μCi of [γ-32P]ATP and GST-IκBα 1–72 wild type (referred as IκBα wt) or GST-IκBα 1–72 A32A36 (IκBα 2A) as substrates.

**RNA Preparation and Northern Blot Analysis**

RNA was extracted in TRIzol (Life Technologies), according to the manufacturer’s instructions. 10 μg of total RNA were separated on 1% formaldehyde-agarose gel, transferred onto nylon membrane (Amersham Pharmacia Biotech), and hybridized with a 32P-radiolabeled probe corresponding to full-length human NRP cDNA. Hybridization was carried out at 60 °C in 7% SDS, 0.5 M sodium phosphate, pH 7.0. The membrane was washed successively in 0.1% SDS, 2 × SSC at 25 °C for 5 min and in 0.1% SDS, 0.1 × SSC at 60 °C for 30 min.

**Phosphatase Treatment of Cell Lysates**

Cells were lysed in 100 μl of 1 × CHIRIS supplemented with 10 μg/ml of the protease inhibitors leupeptin, aprotinin, phenylmethylsulfonyl fluoride. 100 μg of total cell extracts were diluted 4 times with 50 μl Tris-HCl, 5 mM DTT, 2 mM MnCl2, 100 μg/ml bovine serum albumin, pH 8.0, solution containing 500 units of λ-phosphatase (Calbiochem) and incubated at 30 °C for 30 min. The reaction was inhibited with the phosphatase inhibitors sodium fluoride (50 μM), β-glycerophosphate (50 mM), and sodium orthovanadate (1 mM).

**Cell Transfection**

HeLa cells were transfected by the DEAE dextran method. The total DNA content in each transfection was adjusted to 10 μg. The Ig(×βL)-LUC reporter has been described previously (26). In the control, the cells were transfected with the appropriate empty parental expression vector pCDNA3. 24 h after transfection, LPS was added 5 μg before transfection, the cells were lysed for 10 min in 500 μl lysis buffer (25 mM Tris phosphate, pH 7.5, 8 mM MgCl2, 1 mM DTT, 1% Triton, 15% glycerol). Cell debris was removed by centrifugation at 13,000 r.p.m. at 4 °C for 5 min. The whole cell extract was used to measure luciferase activity in the lysis buffer containing 1 μM nuciferin (Roche Molecular Biochemicals) and 20 μM ATP.

**NF-κB Reporter Assays**

1.3E2 cells were transfected by the DEAE dextran method. The total DNA content in each transfection was adjusted to 10 μg. The Ig(×βL)-LUC reporter has been described previously (26). In the control, the cells were transfected with the appropriate empty parental expression vector pCDNA3. 24 h after transfection, LPS was added 5 μg before transfection, the cells were lysed for 10 min in 500 μl lysis buffer (25 mM Tris phosphate, pH 7.5, 8 mM MgCl2, 1 mM DTT, 1% Triton, 15% glycerol). Cell debris was removed by centrifugation at 13,000 r.p.m. at 4 °C for 5 min. The whole cell extract was used to measure luciferase activity in the lysis buffer containing 1 μM nuciferin (Roche Molecular Biochemicals) and 20 μM ATP.

**Immunocomplexes Kinase Assay**

Cells were lysed with CHIRIS buffer as described above. 1 mg of proteins from the lysates were incubated with 5 μl of the NEMO or NRP antibody. The immunoprecipitations were performed as described above, except that the immunoprecipitates were washed 3 times with 1 × CHIRIS followed by 3 washes with a kinase buffer containing: 10 μM Hapes, pH 7.5, 10 mM MgCl2, 100 μM Na3VO4, 20 μM β-glycerophosphate, 10 μM DTT, 50 mM NaCl. The kinase reactions were conducted as described (20) for 30 min at 30 °C in the kinase buffer, in the presence of 12.5 μCi of [γ-32P]ATP and GST-IκBα 1–72 wild type (referred as IκBα wt) or GST-IκBα 1–72 A32A36 (IκBα 2A) as substrates.
antibody (Sigma) diluted 1:200. After being washed in PBS, coverslips were mounted on slides with Mowiol, and cells were examined with a Leica DMRXA-HC microscope.

Preparation of S100 Extracts and Gel Filtration Analysis

Fifty million 293T cells were washed in PBS and resuspended in 500 µl of 50 mM Tris, pH 7.5, and 1 mM EDTA. Cells were lysed by 30 passages through a 26-gauge needle. After centrifugation for 10 min at 1,500 rpm, the supernatant was recovered and complemented with 1 mM DTT, 0.025% Brij 35, and a mixture of proteases and phosphatases inhibitors. S100 were prepared by centrifuging the cytoplasmic extracts for 30 min at 52,000 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 12 (Amersham Pharmacia Biotech) precalibrated with aldolase (158 kDa), catalase (232 kDa), ferritin (440 kDa), and thyroglobulin (669 kDa). Five-hundred µl fractions were recovered and directly analyzed by Western blotting with anti-NEMO and anti-NRP antisera.

In Gel Kinase Assay

Cells were lysed and proteins were immunoprecipitated as described above. Immunoprecipitates were applied to a 10% polyacrylamide-SDS gel which had been polymerized in the presence of 0.5 mg/ml myelin basic protein (bovine brain, Sigma). Following electrophoresis, the gel was subjected to several rounds of denaturation and renaturation. For the denaturation procedure, the gel was washed twice for 30 min with a 50 mM Hepes, pH 7.6, solution containing 20% isopropl alcohol, and twice for 30 min with buffer A (50 mM Hepes, pH 7.6, 5 mM b-mercaptoethanol), and finally twice with 8 M urea in buffer A. Then the gel was renatured progressively at 4 °C by washing the gel once in a 3 M urea solution in buffer A containing 0.025% Tween 20 for 15 min, once in a 1.5 M urea solution in buffer A containing 0.025% Tween 20 for 15 min, and once in a 0.75 M urea solution in buffer A containing 0.025% Tween 20 for another 15 min. For the kinase reaction, the gel was preincubated in kinase buffer (20 mM Hepes, pH 7.6, 20 mM MgCl₂) at 30 °C for 30 min. After denaturation and renaturation, the kinase reaction was initiated by transferring the gel to the kinase buffer containing 10 µM ATP, 20 µM of CPM, and 100 µCi of [γ-32P]ATP. The gel was then washed 4 times for 15 min with trichloroacetic acid/PP, (5% trichloroacetic acid, 1% NaPP₃), dried, and exposed to x-ray film.

RESULTS

NRP Is Structurally Homologous to NEMO—Our previous results have shown that NEMO-deficient cell lines are refractory to all tested NF-κB-activating stimuli, suggesting that IKK is the unique complex involved in NF-κB signaling (20, 27). However, the possibility exists that kinase complexes different from the IKK complex might exhibit a tissue-restricted expression, or might respond to uncharacterized NF-κB activators. We recently showed that NEMO-deficient cell line 1.3E2 was not responsive to LPS stimulation, whereas transfection of the parental cell line with a vector expressing NEMO rescued NF-κB activation under the same conditions, suggesting that NRP could not replace NEMO in NF-κB signaling (20). In contrast to NEMO, transfection of NRP did not result in NF-κB activation under the same conditions, suggesting that NRP could not replace NEMO in NF-κB signaling. We recently showed that the zinc finger region of NEMO is essential for its activity, and that its deletion strongly reduces its ability to complement the 1.3E2 cell line (Fig. 2A).2 As the zinc finger domains of NRP and NEMO are boxed. The additional amino acids in NRP, named as insert in the text, are indicated by arrows.

in 1.3E2 resulted in a 30–40-fold increase in NF-κB activity following LPS stimulation, whereas transfection of the parental expression vector had no effect (Fig. 2A). In contrast to NEMO, transfection of NRP did not result in NF-κB activation under the same conditions, suggesting that NRP could not replace NEMO in NF-κB signaling. We recently showed that the zinc finger region of NEMO is essential for its activity, and that its deletion strongly reduces its ability to complement the 1.3E2 cell line (Fig. 2A).2 As the zinc finger domains of NRP and NEMO are very similar (Fig. 1), we tested the possibility that the zinc finger domain of NRP could functionally replace that of NEMO. A hybrid molecule consisting of the NRP zinc finger fused to the rest of the NEMO molecule (NEMO ΔZF-NRP ZF) could activate NF-κB to the same extent as wild-type NEMO, suggesting a possible functional similarity between the two molecules.

NRP Is Not Associated with NEMO or IKKβ—The significant homology between NEMO and NRP led us to investigate whether NRP was associated with the IKK complex. To examine this possibility, E29.1 T cell hybridoma lysates were subjected to immunoprecipitation with normal rabbit serum as a control (Fig. 2B, lane 1), NRP antibody (lane 2), or NEMO antibody (lane 3). As described previously (20), immunoprecipitat

Fig. 1. Sequence alignment of NRP and NEMO. Alignment of human NRP and NEMO protein sequences using the single letter code. Identical residues are written in bold. Similar structural predicted regions are indicated by CC1 for coiled coil 1, CC2 for coiled coil 2, and ZF for zinc finger. The amino acids of the putative leucine zipper in NRP and NEMO are boxed. The additional amino acids in NRP, named as insert in the text, are indicated by arrows.
tation of NEMO followed by immunoblotting for IKKβ demonstrated the association between these proteins (lane 3); however, we did not find IKKβ in NRP immunoprecipitates (lane 2) and we could not detect an association between NEMO and NRP by immunoprecipitating one of these two proteins followed by immunodetection of the other (lanes 2 and 3). Taken together, these results show that NRP is not associated with NEMO, IKKβ, IKKα (data not shown) and is probably not a component of the IKK complex.

**NRP Is Part of a High Molecular Weight Complex Different from the IKK Complex**—Since NEMO is an essential component of the 600–800-kDa kinase complex that phosphorylates IκB, we investigated whether NRP is also present in a high molecular mass complex. S100 extracts were prepared from 293T cells and fractionated on a Superose 6 gel filtration column. The fractions were analyzed by Western blotting using antibodies directed against NRP and NEMO. As shown in Fig. 2C, NRP is present in a high molecular mass complex ranging from 400 to 700 kDa (lanes 7–10), whereas the larger IKK complex containing NEMO migrates, as expected, at 600 to 800 kDa (lanes 9–11). These results suggest that these two proteins are essentially present in different complexes.

**NRP Is Not Associated with an IκBα Kinase Activity**—The results presented above demonstrate that NRP is not associated with known IκB kinases but, they do not address whether NRP is associated with other putative IκB kinases. In order to elucidate this point, NEMO and NRP were immunoprecipitated from resting or stimulated cells. The immune complexes were assayed for phosphorylation of a glutathione S-transferase (GST) fusion protein containing the amino-terminal part of IκBα in the wild type context (IκBα) or mutated on its two phosphorylation sites (IκBα 2A). In Fig. 2D, top panel, Jurkat cells were stimulated for 10 min with PMA and ionomycin (lanes 3 and 4), anti-CD28 (lanes 5 and 6) or both (lanes 7 and 8). As expected, NEMO was found to be associated with an inducible kinase activity as highlighted by IκBα phosphorylation (compare lanes 3, 5, 7 to lane 1). This activity was directed against the phosphorylation sites of IκBα since IκBα 2A was not phosphorylated (lanes 2, 4, 6, and 8). This phosphorylation was maximum when the cells were co-stimulated with PMA, ionomycin, and anti-CD28 (compare lanes 7 to lanes 3 and 5). In contrast, we could not observe any kinase activity associated with NRP (lanes 9–16). In the bottom panels, we have evaluated the effect of PMA and TNFα treatment on NEMO and NRP kinase activity in HeLa and E29.1 cells. We could observe an inducible kinase activity associated with NEMO after TNFα stimulation (compare lanes 5 and 1) and to a lesser extent after PMA stimulation (compare lanes 3 and 1); however, we could not observe any kinase activity associated with NRP. These data indicate that NRP, in contrast to NEMO, is not associated with an IκBα kinase activity.

**Effect of Different Stimuli on NRP Expression**—In order to investigate the effect of different NF-κB stimuli on NRP expression, 70Z/3 proB cells were stimulated for the indicated time with PMA. As depicted in Fig. 3, top panel, precipitation of NRP followed by immunoblotting with the same antibody demonstrated an increase in the appearance of a slower migrating form of the molecule after 15 min of PMA stimulation (lanes 2–5). This upper band progressively returned to basal level after 1 h of stimulation (lanes 4 and 5). We then tested whether other stimuli could also increase the amount of the upper band. 70Z/3 cells were subjected to IL-1 (middle panel) or LPS stimulation (bottom panel), two other stimuli able to activate NF-κB in this cell line; we could not detect any effect on NRP expression following these treatments. To evaluate whether similar events could occur in T cells, the murine hybridoma E29.1 was subjected to PMA treatment (Fig. 3B, top panel). We could observe a strong increase in the appearance of the slow migrating band after 5 min of stimulation (compare lanes 2 and 1). However, TNFα treatment of E29.1 cells did not result in any change in NRP expression (bottom panel), although prolonged treatment with this cytokine strongly increased the level of the NRP mRNA (see below).

**PMA Induces Phosphorylation of NRP**—We then investigated whether this slowly migrating band represents a hyperphosphorylated form of NRP. E29.1 cells were left untreated or were stimulated for 30 min with PMA and then lysed with Nonidet P-40 containing buffer (Fig. 4, lanes 1 and 2). Following this treatment, cell extracts were treated with λ-phosphatase (lanes 3 and 4) or with λ-phosphatase plus phosphatase inhibitors (lanes 5 and 6). PMA treatment induced the appearance of the upper band (lane 2), which disappeared following phosphatase treatment; this disappearance could be blocked by phosphatase inhibitors (lane 4). This clearly demonstrates that the upper bands represent a hyperphosphorylated form of NRP.

**Stability of NRP Expression and Phosphorylation**—To evaluate the half-life of NRP in stimulated and non-stimulated cells, we used the protein synthesis inhibitor cycloheximide (Fig. 5, top panel). We first treated E29.1 T cells with cycloheximide alone for the indicated period of time (lanes 7–12). This treatment had no effect on the level of expression of NRP until 6.5 h, indicating that NRP has a rather long half-life. In parallel, E29.1 cells were subjected to PMA stimulation (lanes 1–6); this resulted in the appearance of the slowly migrating form described above, and to the slow accumulation of the fast migrating band. The cells were then pretreated with cycloheximide for 30 min and then incubated with cycloheximide and PMA during the indicated times. Inhibition of protein synthesis did not interfere with the appearance of the upper band, but resulted in an accelerated decay of both the slow and fast migrating forms of NRP (compare lanes 13–18 and 1–6). These results suggest that PMA treatment decreases the half-life of NRP.

Since NRP was still phosphorylated after 6 h of PMA stimulation (Fig. 5, lane 6), we then asked whether NRP required an ongoing phosphorylation signal or whether its phosphorylation was stable. To this end, E29.1 T cells were treated as described above with PMA and, after 30 min of treatment (lanes 2 and 7), half of the cells were left with the inducer (lanes 3–5) while the other half were washed twice and resuspended in fresh medium without PMA (lanes 8–10). Following this “chase” of PMA, the phosphorylated band of NRP only slightly decreased (compare lanes 8–10 to lanes 3–5), suggesting that the phosphorylation of NRP is rather stable even when PMA is removed from the medium.

K-252a and Stauroporin Inhibit the Phosphorylation of NRP—In order to characterize the type of kinase leading to NRP phosphorylation, we tested the effect of different kinases inhibitors. As phorbol esters activate protein kinase C (PKC), we first investigated the effect of various PKC inhibitors on the basal or PMA-induced NRP phosphorylation (Fig. 6). Pretreating the cells with GFX (lanes 3 and 4), Gö6976 (lanes 5 and 6), Rottlerin (lanes 7 and 8), K-252b (lanes 11 and 12), H7 (lanes 13 and 14), or calphostin C (lanes 15 and 16) showed no effect. Conversely, treatment of the cells with another PKC inhibitor, K-252a, belonging to the family of alkaloid toxin kinase inhibitors, caused a complete inhibition of PMA-induced as well as basal NRP phosphorylation (compare lanes 9 and 10). As K-252a is also a potent inhibitor of CaM kinase II and protein kinase A we evaluated the effect of additional inhibitors or activators of these kinases. Fig. 6, middle panel shows that the
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A

Fold activation

Control NEMO NRP NEMO ΔZF NEMO ΔZF-NRP ZF

B

IP: NRS NRP NEMO

IKKβ α IKKβ

NRP α NRP

NEMO α NEMO

C

load 440 kDa 669 kDa

NRP α NRP

NEMO α NEMO

D

IP

NEMO

PMA+Iono

anti-CD28

IκBα wt

IκBα 2A

Jurkat

NEMO

NRP

32P GST IκBα wt/2A

HeLa

E29.1

32P GST IκBα wt/2A
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Effect on PMA-induced NRP phosphorylation, suggesting that CaM kinase II is not involved in NRP phosphorylation. In addition, Bt,cAMP, a potent cell-permeable activator of the cAMP-dependent protein kinase (PKA) (lanes 13 and 14) and the pertussis toxin (lanes 15 and 16), which increases intracellular cAMP production increase, were also ineffective in blocking the effect of PMA on NRP. We then evaluated the effect of staurosporin (lower panel, lanes 3 and 4), a potent inhibitor of protein kinases, including CaM kinase, myosin light chain kinase, PKA, PKC, andPKG; genistein (lanes 5 and 6), a protein-tyrosine kinase inhibitor and thapsigargin (lanes 7 and 8), a cell permeable tumor promoting sesquiterpene lactone that releases Ca$^{2+}$ by inhibiting endoplasmic reticular Ca$^{2+}$-ATPase. The cells were also treated with two MAP kinase inhibitors, PD098059 (lanes 9 and 10) andSB203580 (lanes 11 and 12). Fig. 6, lower panel, shows that staurosporin weakly inhibits NRP phosphorylation (compare lanes 4 and 2), genistein has no effect on NRP phosphorylation (lane 6), whereas thapsigargin increases the basal phosphorylation of NRP (compare lanes 7 and 1), suggesting that NRP phosphorylation is Ca$^{2+}$-dependent. Finally, it is unlikely that the MAP kinase kinase and p38 play a role in PMA-induced NRP phosphorylation since this event was not modified by PD098059 orSB03580.

In order to determine the sensitivity of PMA-induced NRP phosphorylation to K-252a and staurosporin, we have titrated their effect. We observed that K-252a and staurosporin are efficient in the range of 0.2–2 and 1 μM, respectively (data not shown).

NRP Is an Interferon-inducible Protein—As interferon is important for many biological responses, we investigated whether NRP is also inducible by interferon. E29.1 cells were treated with interferon γ for different periods of time (Fig. 7A, left panel). We could not observe any effect on NRP expression at the early time points (lanes 3–8), but interestingly after 18 h of treatment, the amount of NRP was significantly increased (lane 2). The same effect could be observed in HeLa cells (right panel, compare lanes 1 and 2). A similar increase could also be observed by immunofluorescence reactions in the presence of [γ$^{32}$P]ATP and 1 μg of GST-LxBα 1–72 wild type (referred as LxBα wt) or GST-LxBα 1–72 A32A36 (LxBα 2A) as substrates. The position of the phosphorylated substrates are indicated on the right.

**Fig. 2.** A, NRP does not complement the NEMO-deficient cell line 1.3E2. The NEMO-deficient cell line 1.3E2 was transiently transfected with the indicated plasmids together with an (Igα3-Luc reporter plasmid. 24 h after transfection the cells were transfected with LPS for 5 h. Cellular extracts were prepared and the luciferase activity was measured. The bars represent the standard variation in at least three experiments. NEMO ΔZF, deletion of the zinc finger of NEMO; NEMO ΔZF-NRP ZF, NEMO with the NRP zinc finger replacing its own zinc finger. B, NRP is not associated with IkKβ and NEMO. 1 μg of protein from whole cell lysate were subjected to immunoprecipitation with preimmune serum (lane 1), anti-NRP (lane 2), or anti-NEMO (lane 3). The transferred Immobilon membrane was cut in 3 parts before immunoblotting. The different antibodies used to blot the corresponding pieces of the membrane are shown on the right. The position of IkKβ, NRP, and NEMO are indicated on the right and the position of the heavy chain of IgG (Ig) is shown on the left. C, gel filtration analysis of NRP and NEMO in 293T cells. S1000 extracts were prepared as described under “Materials and Methods” and fractionated through a Superose 6 column. Fractions were subsequently analyzed by Western blotting using antibodies directed against NRP or NEMO. D, NRP is not associated with an LxBα kinase activity. Jurkat, HeLa, or E29.1 cells were either non-stimulated or stimulated for 10 min with the indicated stimuli, then lysed, and NEMO and NRP proteins were recovered by immunoprecipitation. Kinase activity was assayed by immune complex reactions in the presence of [γ$^{32}$P]ATP and 1 μg of GST-LxBα 1–72 wild type (referred as LxBα wt) or GST-LxBα 1–72 A32A36 (LxBα 2A) as substrates. The position of the phosphorylated substrates are indicated on the right.

**Fig. 3.** A, NRP is upshifted following PMA but not IL-1 or LPS treatment in the murine 70Z/3 pre-B cell line. Cells were stimulated for the indicated periods of time with TNFα, IL-1, or LPS, lysed, and subjected to NRP immunoprecipitation followed by immunoblotting with the NRP antibody. The 2 forms of NRP are indicated on the right by arrows. B, NRP is upshifted following PMA but not TNFα treatment in E29.1 T cell hybridoma. Cells were treated with different inducers for the indicated periods of time. After lysis, and immunoprecipitation with NRP antiserum, the proteins were assayed by immunoblotting with anti-NRP antibody. The position of NRP is indicated on the right by an arrow.

calmodulin kinase inhibitors KN-93 (lanes 5 and 6) and KN-62 (lanes 9 and 10) as well as the calmodulin antagonists W7 (lanes 7 and 8) and calmidazolium (lanes 11 and 12) had no...
observed following treatment with interferon α/β (data not shown). This increase on NRP expression could be blocked by cycloheximide, indicating that it is an indirect effect requiring new protein synthesis (data not shown).

In addition to the effect of interferon on NRP expression, Li and colleagues (24) have found that TNFα could also increase the level of NRP mRNA. We therefore tested in HeLa cells whether interferon and TNFα could cooperatively increase NRP expression (Fig. 7B). Indeed, when compared with unstimulated cells (top panel, lane 1), NRP protein expression was up-regulated in cells treated for 18 h with interferon α/β (lane 2), interferon γ (lane 3), and TNFα (lane 4). The level of expression was further increased when the cells were stimulated by a combination of TNFα and either of the two types of interferon (lanes 5 and 6). In order to determine at which level NRP expression is controlled by TNFα and interferon, HeLa cells were assayed for expression of NRP by Northern blot analysis (bottom panel). As described by Li and colleagues (24), two NRP mRNAs were detected. In agreement with the Western blot, an increased amount of these transcripts could be observed in HeLa cells following treatment by interferon α/β (lane 2), interferon γ (lane 3), and TNFα (lane 4), suggesting that these cytokines regulate NRP expression at the transcriptional level. As shown in lanes 5 and 6, TNFα synergizes with interferon to stimulate the transcription of NRP.

NRP Is Associated with the Golgi Apparatus—In order to investigate the subcellular localization of NRP, we performed indirect immunofluorescence experiments using affinity purified antibodies directed against NRP. Li and colleagues (24) have reported that overexpression of NRP in the murine CSHA cell line results in a “bead-like perinuclear structure” of this protein. In our studies we could observe that overexpressed NRP in HeLa cells shows a diffuse distribution all over the cytoplasm with a weak dominant staining in the Golgi apparatus (Fig. 8A). In contrast, we found that endogenous NRP was exclusively associated with the Golgi network (panel B). This result was confirmed by double immunofluorescence with a Golgi marker (data not shown). It is likely that overexpressing NRP resulted in an nonspecific cytosolic localization. We also investigated whether PMA treatment might change the Golgi localization of NRP, as this stimulus triggers the phosphorylation of this protein (Fig. 3). However, we could not observe any change in the subcellular localization of NRP following this stimulation, suggesting that NRP subcellular localization is not influenced by its phosphorylation state (data not shown).

NRP Is Associated with Two Kinase Activities—We have shown in Fig. 2 that NRP is not associated with IKKα or IKKβ. However, if NRP performs a similar function as NEMO in another signaling pathway, one might expect it to associate with kinases. To identify the presence of such kinases, an in gel kinase assay was performed. To this end, E29.1 T cells were activated or not by the addition of PMA for 30 min. Triton X-100 whole cell lysates were subjected to immunoprecipitation using preimmune serum as a negative control (Fig. 9, lanes 1 and 2), NEMO antibody (lanes 3 and 4), or NRP antibody (lanes 5 and 6). The catalytic activity and the size of the putative kinases was then assessed in vitro as described under “Materials and Methods” using an SDS-polyacrylamide gel electrophoresis containing myelin basic protein as an exogenous substrate. No association with kinases could be seen when normal rabbit serum was used for immunoprecipitation (lanes 1 and 2). As expected, the anti-NEMO immunoprecipitate was associated with a kinase activity with a molecular mass of about 85 kDa, which probably corresponds to IKKα and IKKβ. Thus, the 85- and 180-kDa proteins are two kinases that remain to be identified.

In order to investigate if the associated kinase activity is related to the inducible phosphorylation of NRP itself, we performed an in vitro kinase assay using recombinant GST-NRP fusion protein as a substrate. However, we could not detect any PMA-inducible kinase activity directed against GST-NRP, indicating that NRP is not a substrate of its associated kinase activity (data not shown).

**DISCUSSION**

The IKK complex is required for the inducible phosphorylation of IκB proteins on critical serine residues in response to NF-κB activating stimuli. Two kinases, IKKα and IKKβ, are responsible for the activity of this complex. Recently, we cloned...
the NEMO protein by genetic complementation of a Tax-transformed rat fibroblast cell line (5R) unresponsive to all tested NF-κB activating stimuli (20). This protein interacts with IKKβ and is a component of the IKK complex. We demonstrated that the kinase subunits IKKa and IKKβ require NEMO in order to become responsive to upstream signals. In the 5R cell line, NEMO is absent and reconstitution of the cells with this protein completely restores NF-κB activity. Data base searching with the NEMO protein sequence revealed the presence of human EST clones encoding a NRP. This protein shows significant similarities to NEMO in its primary and predicted secondary structure.

Li et al. (23) have isolated a series of proteins called FIPs that bind to the viral protein Ad E3-14.7K. These proteins include FIP-3 which is identical to NEMO (23) and FIP-2 which is identical to NRP (24). Interestingly, both proteins can reverse the protective effect of E3-14.7K on cell line killing induced by TNFα (23, 24). As NRP and NEMO share 53% sequence similarity, it seems likely that these molecules could fulfill a similar function. We first demonstrated that NRP was unable to complement a NEMO-deficient cell line; however, a chimeric molecule made of the NEMO protein with the zinc finger domain substituted with that of NRP could complement this cell line. This suggests the possibility that this domain might be involved in similar functions in the two proteins. We then investigated whether NRP plays a role in NF-κB signaling. We first tested whether NRP is part of the IKK complex. An analysis of the endogenous IKK complex revealed that IKKa, IKKβ, and NEMO co-elute upon gel filtration (20), while NRP elutes in different fractions (Fig. 2C). In co-immunoprecipitation experiments, NEMO was found associated with IKKβ while we could not find an association of NRP with either of these two proteins (Fig. 2B). Immunoprecipitated NRP was unable to phosphorylate recombinant GST-IκBα protein after treatment with several NF-κB inducers (Fig. 2D). The most striking difference between NEMO and NRP is the presence of
NRP expression is increased by TNF.

Cell lysates were analyzed by immunoblotting using NRP antiserum. 22788 cells were treated with murine interferon-γ, HeLa cells were left untreated or treated for 18 h with human interferon-γ, Ig and NRP are indicated on the left. Right. Right panel, HeLa cells were stimulated for 18 h with different inducers, as indicated, lysed, and subjected to NRP immunoblotting. The position of NRP is indicated on the right by an arrow. Bottom panel, Northern blotting analysis. 10 µg of total RNA was used in each conditions. The position of NRP transcripts is indicated on the right.

Fig. 7. A, NRP is an interferon inducible protein. Left panel, E29.1 T cells were treated with murine interferon-γ for the indicated periods of time. Cell lysates were immunoprecipitated using NRP antibody and NRP was detected by Western blotting. The heavy chain of the immunoglobins (Ig) and NRP are indicated on the right. Right panel, HeLa cells were left untreated or treated for 18 h with human interferon-γ. Cell lysates were analyzed by immunoblotting using NRP antiserum. B, NRP expression is increased by TNFα and interferon. Top panel, HeLa cells were stimulated for 18 h with different inducers, as indicated, lysed, and subjected to NRP immunoblotting. The position of NRP is indicated on the right by an arrow. Bottom panel, Northern blotting analysis. 10 µg of total RNA was used in each conditions. The position of NRP transcripts is indicated on the right.

Fig. 8. Subcellular localization of overexpressed and endogenous NRP in HeLa cells. A, HeLa cells were transiently transfected with NRP by electroporation. Twenty-four hours after transfection NRP was detected by indirect immunofluorescence using affinity purified antibody. B, HeLa cells were directly fixed and permeabilized, and NRP was detected with the same procedure as in panel A.

Fig. 9. NRP is associated with two kinase activities. E29.1 cells were stimulated for 30 min with PMA, lysed in Nonidet P-40 containing buffer, and subjected to immunoprecipitations with normal rabbit serum (NRS), NEMO, or NRP antiserum. The enzymatic kinase activities associated with these immunoprecipitates were assessed by in gel kinase assay, in the presence of myelin basic protein. The kinases associated with NEMO and NRP are indicated with arrows. The position of molecular mass markers and of a nonspecific band (NS) are shown on the right.

An insert in the latter protein. The presence of these additional amino acids led us to speculate that it could prevent NRP interaction with IKKβ. However, in vitro translation of a deletion mutant of NRP lacking the insert gave rise to a protein which was unable to interact with IKKβ when co-translated. Furthermore, this NRP variant was still unable to complement a NEMO-deficient cell line (data not shown).

Whereas our data strongly suggest that NRP cannot substitute for NEMO and is not an integral component of the kinase complex, it still might be a regulator of NF-κB activation. However, transfection of 293T cells with an expression vector for NRP did not result in the transactivation or repression of a κB-dependent reporter gene (data not shown).

The possibility remains that NRP could be part of another IKK complex, although NRP does not seem to be associated with an κB kinase activity responsive to TNF, PMA, or anti-CD28. Recently, three groups have identified a new IKK-like kinase, IKKε (28–30). This kinase is 27% identical to IKKα and IKKβ and is part of a high molecular weight complex (28). The IKKε-containing complex is different from the classical IKK complex (28). As the activity of IKKε is PMA-inducible and this kinase is not associated with NEMO, it seemed worth considering that NRP could play the same role as NEMO in the IKKε complex. However, our attempts to co-immunoprecipitate NRP and IKKε from extracts of Jurkat and E29.1 cells (stimulated or not with PMA) were unsuccessful, suggesting that these two proteins do not interact, even following stimulation.3

Another IKK-related kinase, TBK1/NAK, showing a strong homology to IKKε, has been identified recently (31, 32). This protein is 48% identical to IKKε, and 30% identical to IKKa and IKKβ. However, this protein seems to exhibit properties of an IKKK, acting upstream of the IKK complex (31). It was tempting to evaluate the participation of NRP in this complex. As observed with IKKε, we could not identify any interaction between NRP and TBK1/NAK.3

While we were testing the effect of different NF-κB inducers on NRP expression, we found that NRP was hyperphosphorylated following stimulation by PMA, but not by the other stimuli tested. Some basal phosphorylation could be observed in certain cell lines which was further increased after PMA treatment. We also noticed that NRP exhibits a long half-life which is reduced after PMA treatment suggesting that the stability of NRP might be controlled by this phosphorylation event. NRP is still inducibly phosphorylated in a NEMO-deficient cell line excluding the role of the IKK complex in this event. We tested

3 K. Schwamborn, R. Weil, and A. Israel, unpublished data.
NRP expression. NRP does not seem to be involved in NF-
κB activation, as further studies on NRP activation. Obviously, one of our goals
ability of an apparently specific inhibitor will greatly facilitate
NEMO together with the functional similarity of their zinc
signaling, and the intriguing question which remains to be
whether PMA treatment results in a modification of the appar-
ent molecular weight of the NRP-containing complex: compar-
ison of fractions from non-induced and PMA-treated 70Z/3 cells
demonstrated that the size of the complex was unaffected by the
stimulation (data not shown).

To further characterize the kinase activity responsible for
NRP phosphorylation, we tested the effect of various kinase
inhibitors. Given that phosphorylation of NRP was induced by
PMA it seemed natural to test a series of commercially avail-
able inhibitors to different PKCs. Although definitive conclu-
sions are difficult to draw from such inhibition studies, they do
nonetheless allow us to tentatively exclude some PKCs are
major candidates as the NRP kinase. For example, GFX had no
effect on the phosphorylation status and as such it seems
unlikely that PKC isozymes α, β1, β2, γ, δ, and ε are involved in
PMA-induced NRP phosphorylation. Similarly, neither
Gö6976, nor Rottlerin were effective, again consistent with a
lack of involvement of PKCs, β1, γ, and δ. In fact, among the
PKC and CaM kinase inhibitors tested (Fig. 6) only K-252a and
staurosporin were able to block PMA-induced phosphorylation
of NRP. Staurosporin inhibits the proteolytically generated cat-
alytic fragment of protein kinase C while having no effect on
the binding of phorbol esters to the regulatory domain (33).
This is consistent with the report that in vitro K-252a competes
with ATP but not with phospholipid or Ca\(^{2+}\) (34). The avail-
ability of an apparently specific inhibitor will greatly facilitate
future studies on NRP activation. Obviously, one of our goals
will be the identification of the PMA-inducible kinase respon-
sible for phosphorylation of NRP.

The present work shows the complexity of the regulation of
NRP expression. NRP does not seem to be involved in NF-κB
signaling, and the intriguing question which remains to be
solved is to identify the pathway in which this protein is in-
volved. The strong sequence homology between NRP and NEMO
together with the functional similarity of their zinc
finger suggest that NRP might have a similar role as NEMO in
a different signaling pathway. The additional finding that NRP
interacts with two kinases (Fig. 9) indicates that NRP could
fulfill, like NEMO, a function in the assembly and activity of
these kinases.

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