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Involvement of Double-stranded RNA-activated Protein Kinase in the Synergistic Activation of Nuclear Factor-κB by Tumor Necrosis Factor-α and γ-Interferon in Preneuronal Cells*

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Tumor necrosis factor-α (TNF-α) and γ-interferon (IFN-γ) cooperate during a variety of biological responses and ultimately synergistically enhance the expression of genes involved in immune and inflammatory responses. Recently, we demonstrated that IFN-γ can significantly potentiate TNF-α-induced nuclear factor (NF)-κB nuclear translocation in neuronal derived and endothelial cell lines. The mechanism by which these two cytokines exert their synergistic effect on NF-κB involves the *de novo* degradation of the NF-κB inhibitor, IκBβ. The double-stranded RNA-dependent kinase PKR is IFN-inducible and has been implicated in the activation of NF-κB; therefore, we examined the possibility that PKR may play a role in the synergistic activation of NF-κB during TNF-α/IFN-γ cotreatment. The PKR inhibitor 2-aminopurine (2-AP) inhibited TNF-α/IFN-γ-induced NF-κB nuclear translocation in neuronal derived cells but not in endothelial cells. The induced degradation of IκBβ, which is normally observed upon TNF-α/IFN-γ cotreatment, was blocked completely by 2-AP in neuronal derived cells. Also, 2-AP treatment or overexpression of a catalytically inactive PKR inhibited the TNF-α/IFN-γ-induced synergistic activation of κB-dependent gene expression. Our results suggest that the signal generated by IFN-γ during TNF-α/IFN-γ cotreatment may require PKR to elicit enhanced NF-κB activity, and this signal may affect the stability of the IκBβ protein.

The transcription factor nuclear factor-κB (NF-κB)[1] is activated by a variety of stimuli including cytokines, mitogens, cellular stress, and bacterial or viral products (for review, see Refs. 1–5). The family of mammalian NF-κB transcription factors consists of at least five distinct members: c-Rel, p50 (NF-κB1), p52 (NF-κB2), p65 (RelA), and RelB, which form a variety of active homo- and heterodimers (for review, see Refs. 1–5). Classic NF-κB exists as a p50-p65 heterodimer that is sequestered in the cytoplasm by inhibitor proteins collectively referred to as inhibitors of kappa B (IκBs) (for review, see Ref. 4). The two major forms of IκB are IκBα and IκBβ. Upon stimulation, an activated IκB kinase (IKK) complex (6–9) phosphorylates the IκB proteins, which targets these inhibitor proteins for ubiquitination and degradation (10–13). This process allows NF-κB to translocate to the nucleus and regulate gene-specific transcription. Structurally, IκBα and IκBβ are similar, and both interact with p65- and c-Rel-containing dimers through similar binding domains (14). Additionally, both forms of IκB are phosphorylated on analogous serine residues by the activated IKK complex (6). However, IκBα is characteristically involved in the transient activation of NF-κB, whereas IκBβ has been implicated in the persistent activation of NF-κB (14–17). There is also evidence that the stimuli that ultimately target the IκBs for degradation may differ, although this may be cell type-specific or may depend on the concentration of the inducer (14, 15, 18).

The pleiotropic cytokines tumor necrosis factor-α (TNF-α) and interferon (IFN) can function together to coregulate gene expression synergistically in a variety of cell lines. Typically, the coregulatory effects involve the independent activation of NF-κB by TNF-α (for review, see Ref. 4) and of IFN-responsive factors by IFNs (for review, see Refs. 19 and 20), permitting these transcription factors to bind their unique sites within the promoters of target genes such as MHC class I, ICAM-1, VCAM-1, inducible iNOS, interleukin-6, and interleukin-8 (21–26). Recently, we reported that IFN-γ, which typically does not activate NF-κB, synergistically enhances TNF-α-induced nuclear translocation of p50-p65 NF-κB heterodimers and synergistically activates κB-dependent gene expression (27). We also demonstrated that the mechanism for this synergistic activation involved the *de novo* degradation of the IκBβ protein and that the TNF-α/IFN-γ coactivation of NF-κB in PC12 cells is sensitive to the protein tyrosine kinase inhibitor genistein (27).

An important signal transduction molecule that is targeted by IFNs is the double-stranded RNA (dsRNA)-activated protein kinase (PKR). This serine/threonine kinase was first discovered as a translation inhibitor because of its ability to phosphorylate and deactivate the translation initiation factor, eIF-2 (for review, see Ref. 28). It plays a role in cellular antiviral responses and growth control and is a candidate tumor supressor gene (for review, see Refs. 29 and 30). PKR is IFN-inducible, is present at low levels in most cells, and is found in the nucleus as well as in the cytoplasm (31). Recent evidence indicates that activation of NF-κB by dsRNA, but not by TNF-α or interleukin-1β, may involve PKR and that PKR may phosphorylate IκBα *in vitro* (32–35). Therefore, we examined the pos-

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[1] The abbreviations used are: NF-κB, nuclear factor kappa B; IκB, inhibitor of kappa B; IKK, IκB kinase; TNF-α, tumor necrosis factor-α; IFN, interferon; MHC, major histocompatibility complex; PKR, double-stranded RNA-activated protein kinase; dsRNA, double-stranded RNA; eIF-2, eukaryotic initiation factor-2; 2-AP, 2-aminopurine; EMSA, electrophoretic mobility shift analysis; wtPKR, wild-type PKR, mutPKR, mutant PKR; *Pkr*$^{0/0}$ MEFs, mouse embryo fibroblasts devoid of functional PKR; *Pkr* $^{+/+}$ MEFs, wild-type mouse embryo fibroblasts.

sibility that PKR may be involved in TNF-α/IFN-γ-induced synergistic activation of NF-κB.

In this report, we provide evidence supporting a role for PKR involvement in the synergistic activation of NF-κB by TNF-α/IFN-γ cotreatment in the preneuronal derived cell line, PC12. The PKR inhibitor 2-aminopurine (2-AP) (36, 37) blocks synergistic TNF-α/IFN-γ-induced NF-κB nuclear translocation. The requirement for PKR may be specific for cells of neuronal origin because 2-AP was able to block the synergy in another neuronal derived cell line (B12), but not in an endothelial cell line (EA.hy926). The synergistic activation of κB-dependent gene expression can be inhibited by 2-AP or by the overexpression of a catalytically inactive, dominant negative form of PKR. Also, 2-AP inhibits the *de novo* degradation of IκBβ observed during TNF-α/IFN-γ cotreatment in PC12 cells and B12 cells but does not affect the normal pattern of IκBα degradation. Therefore, the mechanism by which the IFN-inducible kinase PKR functions in this system may involve targeted phosphorylation and degradation of IκBβ. These data indicate a novel role for PKR in the activation of NF-κB.

EXPERIMENTAL PROCEDURES

Cell Culture and Treatments—The rat preneuronal adrenal pheochromocytoma cell line, PC12 (CRL 1721, American Type Culture Collection, Rockville, MD) (38), was maintained in Dulbecco's modified Eagle's medium F-12 supplemented with 10% fetal bovine serum and antibiotics. The central nervous system-derived rat preneuronal cell line B12 (gift of Dave Schubert, The Salk Institute, La Jolla, CA) (39) was maintained in Dulbecco's modified Eagle's medium H supplemented with 10% fetal bovine serum and antibiotics. The human vascular endothelial cell line EA.hy926 (gift of Cora-Jean S. Edgell, University of North Carolina, Chapel Hill) (40) was maintained in Dulbecco's modified Eagle's medium H supplemented with 10% fetal bovine serum, 1× hypoxanthine-aminopterin-thymidine medium supplement (Boehringer Mannheim), and antibiotics.

Cells were incubated for the times indicated under "Results" with 0.025–10 ng/ml human recombinant TNF-α (Boehringer Mannheim), 50–100 units/ml rat recombinant IFN-γ (Life Technologies, Inc.), or 10 mM 2-AP (Sigma).

Nuclear and Cytoplasmic Extracts—The day before treatment, cells were plated in 10 ml of complete media in 100-mm tissue culture plates at 1×10^7 cells/plate (PC12), 1×10^6 cells/plate (B12), or 2×10^6 cells/plate (EA.hy926). After treatment, nuclear and cytoplasmic extracts were made using a procedure described previously (27). Briefly, cells were washed with phosphate-buffered saline, scraped from plates, transferred to microcentrifuge tubes, and lysed on ice in 3 pellet volumes of cytoplasmic extraction buffer (10 mM Hepes, pH 7.6, 60 mM KCl, 1 mM EDTA, 0.1% Nonidet P-40, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 2.5 μg/ml each of aprotinin, leupeptin, and pepstatin). Nuclei were pelleted, and cytoplasmic supernatants were transferred to fresh tubes. Nuclei were washed with 100 μl of extraction buffer without Nonidet P-40 and then repelleted. Supernatants were discarded, and nuclear pellets were resuspended by vortexing in 2 pellet volumes of nuclear extraction buffer (20 mM Tris, pH 8.0, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 25% glycerol, and 2.5 μg/ml each of aprotinin, leupeptin, and pepstatin) in which the final salt concentration was adjusted to ~400 mM NaCl. All cytoplasmic and nuclear extracts were cleared and transferred to fresh tubes. Next, glycerol was added to the cytoplasmic extracts to a final concentration of 20%, protein concentrations were determined by the Bradford assay using the Bio-Rad protein assay dye reagent (500-0006), and all extracts were stored at −70 °C until analyzed.

EMSAs—Electrophoretic mobility shift assays (EMSAs) were performed as described previously (27). Briefly, equal amounts of nuclear extracts were incubated for 15 min at room temperature with a ^{32}P-labeled probe containing a κB site from the class I MHC promoter (41, 42) in binding buffer (10 mM Tris, pH 7.7, 50 mM NaCl, 0.5 mM EDTA, 1 mM dithiothreitol, and 10% glycerol) (43) plus 2 μg of poly(dI-dC)·poly(dI-dC) (Amersham Pharmacia Biotech). Complexes were separated in 5% polyacrylamide gels in Tris-glycine-EDTA buffer (25 mM Tris, 190 mM glycine, and 1 mM EDTA), dried, and autoradiographed.

Western Blot Analysis—Equal amounts of cytoplasmic extracts were electrophoresed in 10% polyacrylamide-SDS gels and transferred to nitrocellulose membranes (Schleicher & Schuell) (27). The upper half of each membrane was probed with an antibody specific for IκBβ (sc-945, Santa Cruz), and the lower half was probed with an antibody specific for IκBα (100-4167C, Rockland). Specific proteins were visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech).

Transient Transfections and Luciferase Assays—Transient transfection of PC12 cells was accomplished using 20 μl/ml LipofectAMINE reagent (Life Technologies, Inc.) and a total of 6 μg of DNA for each sample. The MHC-NF-κBLuc plasmid contains three tandem repeats of the κB site from the class I MHC enhancer cloned into a luciferase expression vector (44). Luciferase expression vector was a gift of Bill Sugden, University of Wisconsin, Madison. The wild-type PKR expression plasmid (wtPKR) and the catalytically inactive $Lys^{296} \rightarrow Arg$ mutant PKR expression plasmid (mutPKR) were described previously (32). PC12 cells were plated in 60-mm tissue culture plates (7×10^6 cells/plate) the day before transfection. LipofectAMINE-DNA complexes were allowed to form for 30 min in serum-free medium before being added to plates containing cells plus 2 ml of serum-free medium (27). Cells were incubated with the complexes for 7–8 h, the medium was replaced with medium containing 0.5% serum, and 8 h of cytokine treatment began 36 h after the medium change. Cells were washed, collected, resuspended in 2 pellet volumes of 0.25 M Tris pH 7.5, and subjected to three cycles of freeze/thaw. Samples were cleared, and protein concentrations were determined using the Bio-Rad protein assay dye reagent. Luciferase assays were performed in duplicate on equal amounts of protein using 200 μM D-Luciferin as a substrate (Sigma), and relative light units were determined using an AutoLumat LB953 luminometer (Berthold Analytical Instruments, Inc., Nashua, NH).

Stable Transfectants—PC12 cells stably expressing MHC-NF-κBLuc or its mutated counterpart were obtained by LipofectAMINE cotransfections with the reporter plasmid and pcDNA3 (Invitrogen, Carlsbad, CA), which contains the neomycin resistance gene. 2 days post-transfection, the medium was replaced with complete medium containing 600 μg/ml Geneticin/G418 (Life Technologies) for selection purposes. Fresh G418-containing medium was added every 4–5 days for 2 months, allowing pools of cells stably expressing MHC-NF-κBLuc to grow out. Stably transfected cells were plated (80% confluent in 60-mm plates) the day before treatment. Where indicated, cells were pretreated for 1 h with 10 mM 2-aminopurine before treatment for 8 h with TNF-α and/or IFN-γ. Cells were collected, lysed, and assayed as described for transient transfections (see above).

RESULTS

TNF-α/IFN-γ-induced NF-κB Nuclear Translocation Is Inhibited by 2-AP—Previously, we demonstrated that IFN-γ treatment synergistically enhances TNF-α-induced nuclear translocation of NF-κB in PC12 cells even though IFN-γ, by itself, does not induce NF-κB in these cells (27). In response to dsRNA treatment but not to TNF-α or IL-1β treatment, the Ser/Thr protein kinase PKR can induce NF-κB DNA binding activity, and this may occur following phosphorylation of IκBα (32–35). To determine if IFN-γ-enhanced NF-κB activity involves PKR, PC12 cells were pretreated for 30 min with 2-AP, a selective inhibitor of PKR, which can inhibit PKR autophosphorylation and activation (36, 37), and then were treated with TNF-α and/or IFN-γ. Nuclear extracts were prepared and analyzed by EMSA. As described previously (27), there was very little binding to a consensus κB site with nuclear extracts from untreated PC12 cells (Fig. 1*A*, *lane 1*). Although it has been documented that a 2-h incubation with 10 mM 2-AP can slightly increase NF-κB DNA binding in the human promonocytic cell line U937 (45), we do not detect a change in binding in PC12 cells after 1 or 3.5 h of treatment (compare *lanes 1–3*). Treatment with TNF-α alone for 30 min (*lane 4*) induced binding of one major NF-κB-specific complex that was identified previously as p50-p65 (27). By 3 h of TNF-α treatment the binding activity was reduced significantly (*lane 6*) and returned to basal levels by 16 h (data not shown). Pretreatment with 2-AP did not affect the TNF-α-induced NF-κB DNA binding profile (compare *lane 4* with *5* and *lane 6* with *7*). As expected, treatment with IFN-γ alone or after pretreatment with 2-AP did not induce binding to the NF-κB-specific probe (*lanes 8–11*). Co-

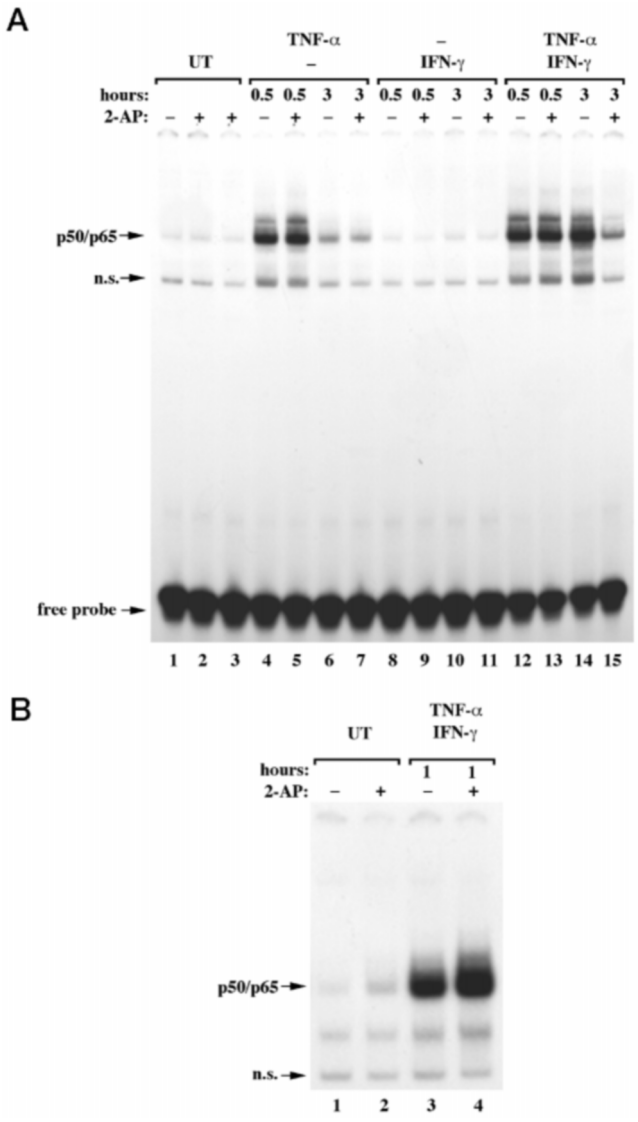

FIG. 1. **2-AP inhibits TNF-α/IFN-γ-induced NF-κB activation in PC12 cells but not in EA.hy926 cells.** EMSAs of equal amounts of protein from nuclear extracts using a probe containing a consensus NF-κB binding site are shown. The time and treatment are indicated above each *lane* (*UT* is untreated). Where indicated, cells treated with cytokine were preincubated with 10 mM 2-AP for 30 min. *Arrows* indicate the major NF-κB-specific band (*p50/p65*), a nonspecific band (*n.s.*), and free probe. *Panel A*, for PC12 cells, TNF-α and IFN-γ concentrations were 10 ng/ml and 100 units/ml, respectively. As a control, cells not treated with cytokine were incubated with 2-AP for 1 h (*lane 2*) or 3.5 h (*lane 3*). *Panel B*, for EA.hy926 cells, TNF-α and IFN-γ concentrations were 25 pg/ml and 100 units/ml, respectively. As a control, cells not treated with cytokine were incubated with 2-AP for 1.5 h (*lane 2*).

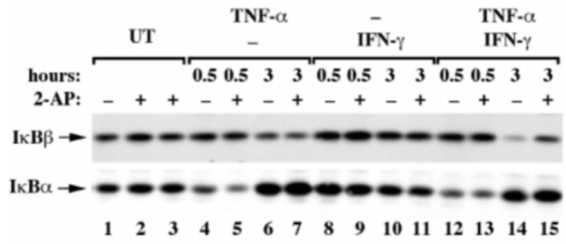

FIG. 2. **2-AP inhibits TNF-α/IFN-γ-induced IκBβ degradation.** Western analyses of equal amounts of protein from PC12 cell cytoplasmic extracts using polyclonal antibodies specific for IκBα or IκBβ are shown. The time and treatment are indicated above each *lane* (*UT* is untreated). TNF-α, IFN-γ, and 2-AP concentrations were 10 ng/ml, 100 units/ml, and 10 mM, respectively. As a control, cells not treated with cytokine were incubated with 2-AP for 1 h (*lane 2*) or 3.5 h (*lane 3*). Where indicated, cells treated with cytokine were preincubated with 2-AP for 30 min (*lanes 5*, *7*, *9*, *11*, *13*, and *15*). *Arrows* indicate each specific IκB protein.

treatment with TNF-α and IFN-γ elicited a striking synergistic effect on κB-specific binding activity after 3 h of cotreatment as reported earlier (compare *lane 6* with *lane 14*) (27). Pretreatment with 2-AP completely blocked the TNF-α/IFN-γ-induced synergy (compare *lanes 14* and *15*). Similar experiments were performed with the central nervous system-derived B12 cell line, and the effects of cytokine treatment with and without 2-AP treatment were nearly indistinguishable from the PC12 cell NF-κB activation profiles (data not shown). In the endothelial cell line, EA.hy926, the TNF-α/IFN-γ-induced activation of NF-κB was not inhibited by 2-AP but was enhanced slightly (Fig. 1*B*, compare *lanes 3* and *4*). Pretreatment with the broad specificity serine/threonine kinase inhibitor staurosporine had no effect on the TNF-α/IFN-γ-induced synergy in either cell type (data not shown). Collectively, the data from these three cell lines indicate that PKR may be involved in the regulatory mechanism for this synergistic response in cells of neural origin.

TNF-α/IFN-γ-induced Degradation of IκBβ Is Inhibitied by 2-AP—Typically, NF-κB is retained in the cytoplasm by inhibitory proteins that are collectively referred to as IκB proteins (for review, see Refs. 3 and 4). In general, NF-κB-inducing stimuli promote the degradation of specific IκB proteins, which allows the release and nuclear translocation of NF-κB subunits. Previously, we demonstrated that costimulation of NF-κB by TNF-α and IFN-γ in PC12 cells requires the *de novo* degradation of IκBβ (27). Therefore, we investigated whether 2-AP affects IκBα or IκBβ protein degradation. Western blot analyses were performed on cytoplasmic extracts collected at the same time as the nuclear extracts that were analyzed for Fig. 1*A*. Incubation for up to 3 h with 2-AP alone had no effect on either IκBα or IκBβ protein levels (Fig. 2, compare *lanes 1–3*). TNF-α treatment for 30 min resulted in degradation of IκBα but not IκBβ protein levels (compare *lanes 1* and *4*), and 2-AP did not inhibit this degradation (compare *lanes 1*, *4*, and *5*). In fact, 2-AP appears to enhance IκBα degradation in the presence of TNF-α, consistent with the slight increase in DNA binding activity observed in Fig. 1*A*, *lanes 4* and *5*. TNF-α was able to lead to a modest reduction in IκBβ levels after 3 h of stimulation, and this was not affected by 2-AP (Fig. 1*A*, *lanes 6* and *7*). As expected, IκBα was resynthesized within 3 h because the expression of IκBα is transcriptionally regulated by NF-κB (*lane 6*) (for review, see Ref. 4). Treatment with IFN-γ either alone or after pretreatment with 2-AP also did not change the level of either IκB protein (*lanes 8–11*). Furthermore, TNF-α/IFN-γ cotreatment caused extensive degradation of IκBβ which corresponds to the synergistic activation of NF-κB shown in Fig. 1 (Fig. 2, *lane 14*). Interestingly, the degradation of IκBβ was inhibited by 2-AP (compare *lanes 14* and *15*), which corresponds to the inhibition of NF-κB activity shown in Fig. 1. Similar experiments were performed with the central nervous system-derived B12 cell line, and the effect of 2-AP on TNF-α/IFN-γ-induced IκBβ degradation was nearly identical (data not shown). We were unable to identify a higher mobility, hypophosphorylated form of IκBβ which has been detected following its initial degradation (17). Also, we have not analyzed the potential of TNF-α and IFN-γ to lead to enhanced degradation of other forms of IκB, such as IκBε. Our data indicate that the degradation of IκBβ in response to TNF-α/IFN-γ cotreatment and therefore the synergistic activation of NF-κB may be PKR-dependent in cells of neuronal origin.

PKR May Play a Role in the Synergistic Activation of κB-dependent Gene Expression by TNF-α/IFN-γ Cotreatment—To

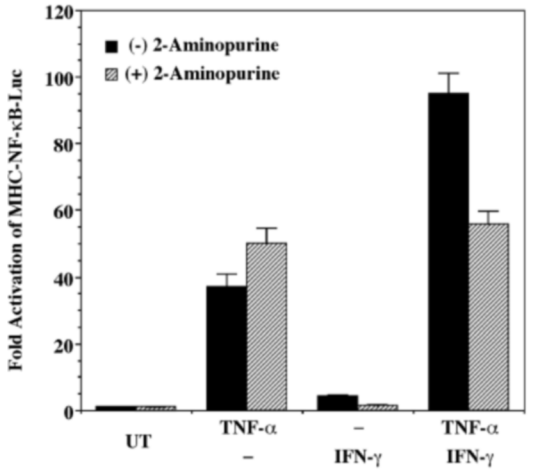

FIG. 3. **2-AP inhibits TNF-α/IFN-γ-induced κB-specific gene expression.** PC12 cells stably expressing a κB-dependent luciferase reporter construct (MHC-NF-κBLuc) were pretreated for 30 min with 10 mM 2-AP or were left untreated. Subsequently, the cells were treated with 10 ng/ml TNF-α, 100 units/ml IFN-γ, or a combination of both for 7 h. Lysates were assayed in duplicate for luciferase activity, and fold activity was determined by dividing the number of relative light units from treated samples by the number of relative light units from untreated (*UT*) samples. The data shown are averages of three independent experiments, and the S.E. of the mean are indicated by *error bars*.

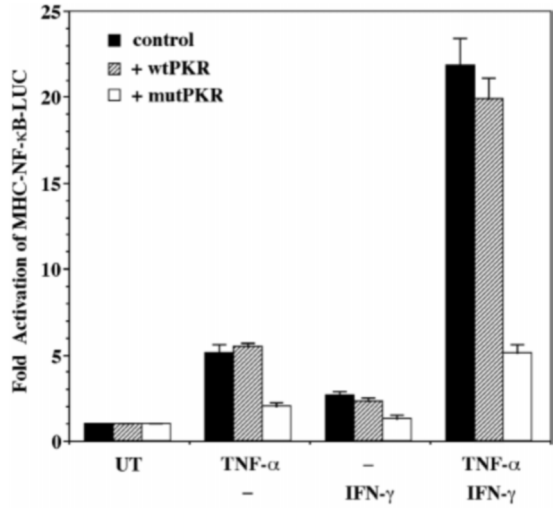

FIG. 4. **MutPKR inhibits TNF-α/IFN-γ-induced κB-specific gene expression.** PC12 cells transiently transfected with a κB-dependent luciferase reporter construct (MHC-NF-κBLuc) alone or in combination with a plasmid expressing wtPKR or a catalytically inactive mutPKR were treated with 10 ng/ml TNF-α, 100 units/ml IFN-γ, or a combination of both for 7 h. Lysates were assayed in duplicate for luciferase activity, and fold activity was determined by dividing the number of relative light units from treated samples by the number of relative light units from untreated (*UT*) samples. The data shown are averages of three independent experiments, and the S.E. of the mean are indicated by *error bars*.

test whether the synergistic activation of NF-κB-dependent transcriptional responses requires PKR activity, we examined the ability of 2-AP to inhibit κB-dependent reporter gene expression. We used cells that were stably transfected rather than transiently transfected with a κB-dependent reporter because transient expression can be affected by 2-AP (45–47). PC12 cells stably maintaining a luciferase reporter construct containing three κB sites cloned in tandem in front of the minimal luciferase promoter (MHC-NF-κBLuc) were treated with TNF-α and/or IFN-γ in the presence or absence of 2-AP. The κB sites conferred a ~35-fold induction of luciferase activity upon treatment with TNF-α, a ~5-fold induction upon treatment with IFN-γ, and a synergistic ~95-fold induction upon cotreatment (Fig. 3). Preincubation with 2-AP significantly reduced the TNF-α/IFN-γ induction of MHC-NF-κBLuc by ~40%, eliminating the IFN-γ-supplied synergism. 2-AP did not nonspecifically affect gene expression (data not shown).

To explore further the requirement for PKR during TNF-α/IFN-γ-induced κB-dependent gene expression, we transiently cotransfected PC12 cells with MHC-NF-κBLuc plus a plasmid that expresses wtPKR or one that expresses mutPKR. MutPKR contains a $Lys^{296} \rightarrow Arg$ mutation which makes it a catalytically inactive kinase (32). The inactive PKR acts as a dominant negative either by competing for an endogenous PKR activator (48, 49) or by forming inactive dimers with endogenous PKR (50, 51). After transfection, we treated the cells with TNF-α and/or IFN-γ and compared the luciferase activity relative to cells that were transfected with the reporter construct alone. WtPKR had little effect on the increased luciferase activity observed after cytokine treatment; however, mutPKR inhibited TNF-α/IFN-γ-induced MHC-NF-κBLuc activity by ~80% (Fig. 4). The expression of mutPKR also decreased TNF-α-induced luciferase activity, suggesting that a minor component of TNF-α signaling may involve PKR and that its inhibitory effect may be on the ability of NF-κB to transactivate gene expression rather than on its ability to translocate to the nucleus. This effect has been documented previously by Kumar *et al.* (32). In summary, these data indicate that IFN-γ enhances TNF-α-induced NF-κB-dependent transcription through PKR.

DISCUSSION

The cooperation between TNF-α and IFN-γ during many biological responses including the regulation of gene expression is well documented (for review, see Refs. 52 and 53), and there are several mechanisms by which these two cytokines can collaborate. For example, cooperation can be achieved by mutual up-regulation of each other's receptors (54–58). In the context of gene expression, the synergy between TNF-α and IFN-γ is thought to be the result of the independent activation of NF-κB by TNF-α and of IFN regulatory factors or signal transducers and activators of transcription by IFN-γ which bind to unique promoter sites and synergistically regulate gene expression. Previously, we reported a mechanism by which these two cytokines can synergistically activate gene expression in an endothelial and a preneuronal cell line (27). In our model, IFNs significantly potentiate the TNF-α-induced nuclear translocation of NF-κB and κB-dependent gene expression. The mechanism for this synergy involves the *de novo* degradation of IκBβ. This is a novel mechanism for NF-κB activation because IFN-γ alone does not activate NF-κB. The net result is the targeted degradation of both IκBα and IκBβ which increases the amount of NF-κB that is free to translocate into the nucleus and therefore synergistically increases κB-dependent gene expression.

What is the signal generated by IFN-γ binding to its receptor which is responsible for enhanced nuclear translocation of NF-κB and the synergistic activation of κB-dependent gene expression during TNF-α/IFN-γ cotreatment? Our data indicate that in cells of neural origin, IFN-γ potentiates the ability of TNF-α to induce NF-κB activity by targeting the serine/threonine kinase, PKR. This dsRNA-activated, IFN-inducible kinase is best known for its role during antiviral responses where, in response to dsRNA, it homodimerizes and autophosphorylates and then phosphorylates and deactivates its primary target, the translation initiation factor, eIF-2. However, PKR has also been implicated in controlling cell growth, cell differentiation, and tumor supression (for review, see Refs. 29 and 30), and there is evidence that it can become phosphoryl-

ated in the absence of viral infection or dsRNA treatment (59). In PC12 and B12 cells, pretreatment with the PKR-specific inhibitor 2-AP completely blocked TNF-α/IFN-γ-induced NF-κB nuclear translocation and reduced κB-dependent gene expression by at least 40% (Figs. 1 and 3). Because it is possible that 2-AP could affect molecules other than PKR (36), we specifically targeted PKR by transfecting cells with a catalytically inactive form of PKR. This dominant negative PKR effectively reduced the level of TNF-α/IFN-γ-induced κB-dependent gene expression to the level observed with TNF-α alone (~80% reduction) (Fig. 4).

The requirement for PKR may be cell type-specific because 2-AP completely blocked the TNF-α/IFN-γ-induced activation of NF-κB in cells of neural origin but not in endothelial cells (Fig. 1). Petryshyn *et al.* have shown that IFN-induced PKR activity does not occur until at least 3 h after treatment (60, 61). This might explain the cell type-specific effect of 2-AP because the synergistic activation of NF-κB occurs in endothelial cells within 15 min to 1 h post-stimulation, whereas the synergy in neuronal derived cells does not occur until later than 2 h post-stimulation (27). Also, 2-AP did not block NF-κB activity induced by TNF-α alone, therefore 2-AP is most likely targeting a signal generated by IFN-γ binding to its receptor.

There is evidence that PKR can affect the activation of NF-κB in mouse embryo fibroblasts isolated from the PKR knockout mouse ($Pkr^{0/0}$ MEFs). In $Pkr^{0/0}$ MEFs, dsRNA-activated NF-κB is reduced compared with levels in wild-type MEFs ($Pkr^{+/+}$ MEFs), but TNF-α-activated NF-κB levels are normal (32). Upon pretreatment with IFN-α or IFN-γ, dsRNA-induced NF-κB activity is restored to normal. Maran *et al.* (34) have used an antisense procedure to decrease selectively the level of PKR activity in cells. In these cells, dsRNA could not activate NF-κB, but the activation of NF-κB by TNF-α was unaffected. Recently, several groups have shown that IκBα may be phosphorylated by PKR *in vitro* in response to dsRNA, but it is not clear whether this occurs *in vivo* (32–35). There is no direct evidence that IκBβ can be phosphorylated by PKR; however, IκBβ has two serine residues that are analogous to the two serines present in IκBα which can be inducibly phosphorylated prior to ubiquitination and degradation (10–12, 14, 18). In an attempt to implicate PKR further during synergistic NF-κB activation, we began to examine the effect of TNF-α/IFN-γ cotreatment in $Pkr^{+/+}$ MEFs and $Pkr^{0/0}$ MEFs. The $Pkr^{+/+}$ MEFs did not exhibit synergistic activation of NF-κB (at least with the concentrations of TNF-α and IFN-γ used to generate synergism in PC12 cells), therefore we were unable to use this cell model for further studies. These results further strengthen our hypothesis that the mechanisms for the synergistic activation of NF-κB will be diverse and cell type-specific.

What is PKR's target during NF-κB activation? A major component of the TNF-α/IFN-γ-induced activation of NF-κB is the mechanistic switch from IκBα degradation to IκBβ degradation, leading to persistent activation of NF-κB. This is similar to previous documentation where IκBα is thought to be involved in the transient activation of NF-κB, whereas IκBβ is targeted during the persistent activation of NF-κB (14–17) Also, it has been proposed that IκBβ can act either as an inhibitor or as a chaperone-like protein. As a chaperone, IκBβ could protect NF-κB from the inhibitory properties of IκBα, and this mechanism may be explained by the differential phosphorylation of IκBβ (17). In our system, using 2-AP to block PKR activity inhibits the *de novo* degradation of IκBβ during costimulation but does not affect the pattern of IκBα degradation. Blocking IκBβ degradation is concomitant with inhibiting the synergistic and prolonged activation of NF-κB. This leads to a model whereby signals generated from TNF-α/IFN-γ cotreatment activate PKR, which in turn either directly or indirectly causes the induced phosphorylation and degradation of IκBβ and consequently the synergistic activation of NF-κB.

There are several possible mechanisms by which TNF-α/IFN-γ cotreatment could target PKR. First, PKR protein levels could be up-regulated by IFN-γ or by TNF-α (62; and for review, see Refs. 29 and 30). However, our previous work using cycloheximide demonstrates that protein synthesis is not required for this TNF-α/IFN-γ synergy, therefore an increase in PKR protein levels cannot account for this response. Second, IFN-γ may lead to an increased activity of PKR, but signals generated by the presence of both cytokines may be required to target the NF-κB·IκBβ complex. Third, signals generated by cotreatment could induce the synthesis of or change the structure of a cellular dsRNA or other PKR activator, which could then activate PKR (59). Also, there is evidence for endogenous proteins that act as cellular PKR inhibitors (63, 64); therefore cotreatment could generate signals that could counteract the inhibitory roles of these proteins. Another possibility is that IFN-γ and/or TNF-α may generate signals that induce phosphorylation and activation of PKR. PKR could then directly phosphorylate IκB proteins and target them for degradation or activate a kinase that is responsible for IκB phosphorylation. Recently, two subunits of the multiprotein complex that forms a functional IKK have been isolated and characterized. Both IKKα and IKKβ are TNF-α-inducible and specifically phosphorylate both IκBα and IκBβ on critical serine residues (6–9). It has been reported that IKK itself may require phosphorylation for activation, although the required kinase(s) has not been identified (7). Potentially, there could be distinct IKKs that are specific for individual IκB proteins *in vivo*, and an IKK which specifically targets IκBβ could be a substrate for PKR. It is also likely that the strength of the signal determines the extent to which NF-κB will be activated. For example, TNF-α can activate IKK but maybe only to limited levels. However, in the presence of signals generated by IFN-γ (such as increased PKR activity) the activity of IKK could be elevated and subsequently increase and/or prolong the activity of NF-κB.

The results presented here are directed at elucidating the mechanisms(s) whereby IFNs impinge on TNF-α-induced activation of NF-κB. Our data indicate a role for the IFN-inducible kinase PKR in this response in cells of neuronal origin. Clearly there are implications that the synergistic activation of NF-κB in neural derived cells may be important in suppressing an apoptotic mechanism. TNF-α and IFN-γ are both potentially apoptotic agents; however, together they potentiate the activation of the anti-apoptotic activity of NF-κB. Although PKR has been implicated in a mechanism for stress-induced apoptosis (65, 66), the net cellular response to TNF-α and IFN-γ may depend on the source(s) and/or strength of the signals that are generated. Therefore, the synergistic activation of NF-κB by TNF-α and IFN-γ during an inflammation response could protect cells of neural origin from death.

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