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Double-stranded RNA-dependent protein kinase activates transcription factor NF-κB by phosphorylating IκB

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ABSTRACT **The induction of interferon (IFN) genes by viruses or double-stranded RNA (dsRNA) requires the assembly of a complex set of transcription factors on responsive DNA elements of IFN gene promoters. One of the factors necessary for regulating IFN-β gene transcription is nuclear factor NF-κB, the activation of which is triggered by dsRNA. It has previously been suggested that the dsRNA-activated p68 protein kinase (PKR) may act as an inducer–receptor, transducing the signal from dsRNA to NF-κB through phosphorylation of the inhibitor IκB. We present direct evidence that PKR can phosphorylate IκB-α (MAD-3) and activate NF-κB DNA binding activity *in vitro*. We further show that dsRNA induces an unusual phosphorylated form of IκB-α. The expression of a transdominant mutant PKR is able to perturb the dsRNA-mediated signaling pathway *in vivo*, suggesting a role for this kinase in IFN-β gene induction.**

Interferons (IFNs) are a family of proteins with distinct biological properties, the most prominent of which is their ability to impair viral replication (1). The antiviral effects of IFN are in part mediated by the action of a double-stranded RNA (dsRNA)-dependent Ser/Thr-protein kinase (PKR) (2, 3). Once activated by dsRNA, PKR autophosphorylates and in turn phosphorylates the α subunit of eukaryotic initiation factor 2 (eIF-2), leading to a decrease in protein synthesis in mammalian cells (4). Indirect evidence from use of the PKR inhibitor 2-aminopurine (2-AP) has led to the suggestion that PKR may be acting as a signal transducer involved in the regulation of dsRNA-activated genes. dsRNA treatment of human MG63 osteosarcoma cells induced the expression of the IFN-β gene, which is specifically blocked at the transcriptional level by 2-AP treatment, whereas constitutive γ-actin mRNA levels were unaffected (5). Furthermore, 2-AP has been shown to block the vesicular stomatitis virus induction of IFN in both mouse L cells and chicken embryo cells (6). This led to the suggestion that PKR may be acting as an inducer–receptor transducing the signal from dsRNA to a transcription factor through a phosphorylation event.

NF-κB is a multisubunit transcription factor comprising p50, p65, and *rel* protooncogene products (7) that has been implicated in the regulation of the IFN-β gene promoter by dsRNA (8–10). In many unstimulated cells, NF-κB is found localized to the cytoplasm as a latent heterodimeric complex bound to its subunit-specific inhibitor IκB (also called MAD-3) (11). dsRNA treatment of 70Z/3 pre-B-lymphocytes leads to the activation and translocation of transcription factor NF-κB to the nucleus, where it binds its cognate DNA element, positive regulatory domain II (PRDII) (11–13). NF-κB in conjunction with transcription factors high-mobility group 1, activating transcription factor 2, IFN regulatory factor 1 (IRF-1), and c-*jun* product activates the IFN-β promoter (14). The mechanism by which NF-κB is activated by dsRNA or virus infection is not known; however, phosphorylation of IκB has been implicated as a means of NF-κB regulation (15–17). Accordingly, it seemed reasonable to ask whether IκB may be a substrate for PKR. Here we show that NF-κB is activated as a result of PKR phosphorylating IκB.

MATERIALS AND METHODS

Purification of Fusion Proteins. Human full-length PKR was expressed as a glutathione *S*-transferase (GST) fusion in the expression vector pGEX-2T and purified from bacteria by using glutathione-Sepharose chromatography (18). IκB-α, p50, and p65 (10–12) were produced in a similar fashion except that the GST portion of these fusion proteins was cleaved with thrombin and removed by centrifugation (the molecular mass of the bacterially produced IκB-α protein was 37 kDa).

***In Vitro* Phosphorylation Assays.** Phosphorylation assays were performed as follows. PKR-GST (0.1 μg) attached to the glutathione-Sepharose bead matrix was incubated in kinase buffer (2) containing 0.015 mCi (1 Ci = 37 GBq) of [γ-^{32}P]ATP for 10 min at room temperature (RT). IκB-α, p50, and p65 were individually added to separate kinase reaction mixtures, and the PKR-GST attached to the Sepharose beads was removed by centrifugation after a 15-min incubation. The supernatants were analyzed by SDS/10% PAGE and autoradiography.

***In Vitro* Activation of NF-κB.** Electrophoretic mobility-shift assays (EMSAs) were performed with whole-cell extracts (WCEs) prepared from mouse SVT2 cells (19) in a binding buffer composed of 8 mM Hepes (pH 7), 8% (vol/vol) glycerol, 20 mM KCl, 4 mM $MgCl_2$, 1 mM sodium phosphate, 0.2 mM EDTA, 0.1 mM ATP, and 0.3 μg of poly(dI-dC)·poly(dI-dC) in a volume of 16 μl. One microgram of SVT2 WCE was incubated with 5–50 ng of each of the following kinases: PKR-GST fusion protein, [Arg^{296}]PKR-GST fusion protein with mutant PKR carrying the Lys-296 → Arg mutation (4), and p43 v-abl tyrosine kinase (20) for 10 min at RT in EMSA binding buffer. PRDII oligonucleotide end-labeled with [γ-^{32}P]ATP was added to each reaction, and the incubation was continued at RT for 15 min (0.1 ng labeled to 30,000 cpm). The NF-κB complex was identified by using DNA competition and antibody mobility-shift assays. The NF-κB complex was activated with the addition of PKR-GST (as described above). DNA oligonucleotide competitors, PRDII (positions −55 to −66 of the human IFN-β gene promoter) (9), and IFN-

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Abbreviations: IFN, interferon; dsRNA, double-stranded RNA; PKR, dsRNA-activated protein kinase; RT, room temperature; GST, glutathione *S*-transferase; mAb, monoclonal antibody; EMSA, electrophoretic mobility-shift assay; PRDI, PRDII, and PRDIII, positive regulatory domains I, II, and III; CAT, chloramphenicol acetyltransferase; WCE, whole-cell extract; ISRE, IFN-stimulated response element; IBF-1, ISRE-binding factor 1; IRF-1, IFN regulatory factor 1; *tk*, viral gene for thymidine kinase; eIF-2, eukaryotic initiation factor 2; TNF-α, tumor necrosis factor α.

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stimulated response element (ISRE; positions −87 to −108 of the human 2-5A synthetase promoter) (19) were added to the binding reactions as indicated. Antibody mobility shifts were performed by activating NF-κB with 50 ng of PKR-GST and then incubating these WCEs with either 1 μl of stock p50 polyclonal antibody or 1 μl of stock PKR monoclonal antibody (mAb) (2) for 10 min at RT before the addition of PRDII probe. The binding reaction was analyzed by 4% PAGE in 0.25× TBE running buffer (1× TBE = 90 mM Tris/64.6 mM borate/2.5 mM EDTA, pH 8.3) followed by autoradiography. p50 antibody (Santa Cruz Biotechnology) is an affinity-purified rabbit polyclonal antibody raised against a peptide encompassing part of the basic p50 sequence and the N-terminal adjacent 11 amino acids of the p105 precursor of the human p50 protein.

***In Vitro* Inactivation of NF-κB.** IκB-α and ISRE-binding factor 1 (IBF-1) (J. Haque, unpublished data) were phosphorylated as described above. NF-κB was activated in 1 μg of SVT2 WCE with 50 ng of PKR-GST. PKR-GST was subsequently removed from the SVT2 WCE by centrifugation. These SVT2 WCEs containing activated NF-κB were treated with either the unphosphorylated or phosphorylated forms of IκB-α and IBF-1 before the addition of the PRDII probe.

dsRNA-Dependent Phosphorylation of IκB-α (MAD-3). Raw 264.7 mouse macrophage cells were incubated in phosphate-free medium with 0.1% fetal calf serum (FCS) for 4 hr. The medium was removed, and 1.5 mCi of [^{32}P]orthophosphate was added with or without 100 μg of poly(rI·rC) (synthetic dsRNA analog) to the above medium and incubated for 4 hr (5×10^6 cells per plate). The cells were washed in HBS (Hepes-buffered saline) and subsequently lysed for 30 min at 4°C in MLB buffer (25 mM Mops, pH 7.0/250 mM NaCl/5 mM EDTA/0.1% Nonidet P-40/1 mM sodium orthovanadate/1 μg of aprotinin per ml/1 μg of leupeptin per ml/50 μg of phenylmethylsulfonyl fluoride per ml). The lysed cells were centrifuged in a Microfuge for 15 min at 4°C, and the supernatant was removed. To the supernatant 100 μl of protein-Sepharose G beads and 1 μg of rabbit polyclonal IκB-α antibody were added. To determine the specificity of binding to IκB-α antibody, 1 μg of bacterially produced IκB-α protein was added to the supernatant antibody-binding reaction. The beads were washed four times with HBS, and 33 μl of the protein-Sepharose G beads was analyzed by SDS/12% PAGE. IκB-α antibody is an affinity-purified rabbit polyclonal antibody raised against a peptide corresponding to amino acids 6–20 mapping within the amino-terminal domain of human IκB-α (MAD-3). Polyclonal antibody for IκB-α (Santa Cruz Biotechnology) was derived from the human MAD-3 protein. This antibody crossreacts with mouse IκB-α protein but does not crossreact with IκB-β, IκB-γ, or pp40, mouse or human.

Transient Transfection Assays. The reporter plasmid was pBI, which contained the NF-κB enhancer site derived from the human tumor necrosis factor α (TNF-α) promoter (positions −635 to −621 of the human TNF-α promoter, single copy, forward orientation) (21) cloned upstream of the minimal viral thymidine kinase gene (*tk*) promoter fused to the bacterial chloramphenicol acetyltransferase (CAT) gene. Mutant [Arg^{296}]PKR carrying the Lys-296 → Arg mutation was constitutively expressed under the cytomegalovirus (CMV) promoter (construct pRC-M). The Rous sarcoma virus vector RSVβ-gal was used to express β-galactosidase (22). The plasmids were transfected by using the DEAE-dextran/chloroquine method as follows. Raw 264.7 cells (5×10^6 cells per plate) were grown in Dulbecco's modified Eagle's medium (DMEM) containing 0.1% FCS for 4 hr and transfected with the different plasmids. pRC, pBI, and pRC-M were transfected at 20 μg; and RSVβ-gal, at 5 μg. The DMEM/FCS medium was removed, and transfection mixtures in PBS containing various plasmids and DEAE-dextran at 0.3 μg/μl in a volume of 400 μl were added to the cells. After a 30-min incubation, the cells were washed three times with phosphate-buffered saline (PBS), incubated with 5 ml of DMEM/FCS containing 100 μM chloroquine for 90 min, washed three times with PBS, and incubated in fresh DMEM/FCS for 12 hr. This medium was removed, fresh DMEM/FCS with or without poly(rI·rC) at 100 μg/ml was added, and the cells were incubated for a further 8 hr. The cells were harvested in 1 ml of TEN (40 mM Tris, pH 7.8/1 mM EDTA/0.15 M NaCl) and centrifuged in a Microfuge for 10 sec at 4°C. The cell pellets were resuspended in 200 μl of TEN, and WCE was prepared by subjecting to freeze/thaw cycles (alternating from a dry-ice ethanol bath to a 37°C bath for three cycles and mixing in a Vortex vigorously between cycles). The lysed cells were centrifuged at 16,000 × *g* for 15 min at 4°C, and the supernatants were used for CAT (non-chromatographic) and β-galactosidase assays as described (22). The latter assay indicated no significant variation in transfection efficiency between plates; therefore, the CAT activity was directly plotted.

RESULTS

To determine if PKR could directly phosphorylate IκB-α, *in vitro* kinase assays were performed with recombinant DNA-derived proteins. PKR was expressed in bacteria as a fusion protein with GST and purified to homogeneity by glutathione-Sepharose chromatography. Recombinant bacterially produced PKR-GST does not show a dsRNA dependence and is inherently active once purified from bacteria. In addition to dsRNA, other polyanionic molecules such as heparin, poly(L-glutamine), dextran sulfate, and chondroitan sulfate are also able to activate PKR *in vitro* (23). However, denaturing and renaturing PKR-GST with guanidine hydrochloride in the presence of dithiothreitol restores the dsRNA-dependent activation (A.K. and B.R.G.W., unpublished observation). mAb-purified PKR is activated by dsRNA and phosphorylates purified mammalian eIF-2 and bacterially produced IκB-α (A.K. and B.R.G.W., unpublished data). Recombinant PKR-GST derived from bacteria does not require dsRNA but phosphorylates both eIF-2 and IκB-α (A.K. and B.R.G.W., unpublished data). Neither mAb-purified PKR nor bacterially produced PKR-GST phosphorylated bacterially produced 2-5A-dependent RNase, c-ets-1, nucleolin, or the Wilms tumor protein (WT1) (data not shown). Therefore, recombinant PKR-GST retains the same substrate specificity and activity as purified mammalian PKR.

The PKR-GST fusion protein was coupled to an insoluble matrix (glutathione-Sepharose beads) and autophosphorylated with [γ-^{32}P]ATP, and exogenous substrates (IκB-α, p50, and p65) were added to the kinase reactions in the presence of [γ-^{32}P]ATP. The PKR-GST fusion protein bound to the insoluble matrix was removed by centrifugation, and an aliquot of the supernatant containing the putative substrate was analyzed by SDS/PAGE and autoradiography. The results (Fig. 1) show that the PKR-GST fusion protein specifically phosphorylated the IκB-α protein, which is indicated by a phosphoprotein migrating in SDS/PAGE with a molecular weight of 37 kDa (which corresponds to the predicted mobility of IκB-α) (11). This band was observed only in the lanes in which the IκB-α protein was present (Fig. 1, lanes 2–4). The PKR-GST fusion protein was unable to phosphorylate the bacterially produced p50 or p65 subunits of NF-κB (Fig. 1, lanes 5 and 6). Furthermore, IκB-α was not phosphorylated when PKR-GST was omitted from the kinase reaction, suggesting that the transfer of [γ-^{32}P]ATP to IκB-α was the result of a PKR-GST-mediated transfer and not due to the nonspecific binding of [γ-^{32}P]ATP to IκB-α (Fig. 1, lane 8). dsRNA-activated mAb-purified mammalian PKR from Daudi cells also directly phosphorylated IκB-α (data not shown).

The functional consequences of IκB-α phosphorylation by the PKR-GST were investigated by using EMSA on WCEs

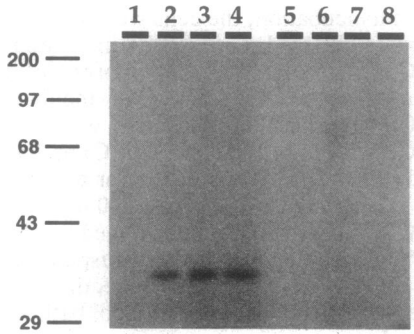

FIG. 1. *In vitro* phosphorylation of IκB-α by PKR-GST. Exogenous substrate was added to the kinase reaction, and substrate phosphorylation was analyzed by SDS/PAGE. Lanes: 1–4, IκB-α was added at 0, 5, 10, and 20 ng, respectively; 5, p50 subunit of NF-κB at 20 ng; 6, p65 subunit of NF-κB at 20 ng; 7, [Arg^{296}]PKR-GST plus 20 ng of IκB-α; 8, IκB-α at 20 ng incubated in kinase buffer and [γ-^{32}]ATP.

from SVT2 cells. We reasoned that if the phosphorylation of IκB-α with purified reagents was biologically meaningful, the PKR-GST should phosphorylate IκB-α bound to NF-κB in a WCE and release the active form of NF-κB, allowing it to bind its cognate DNA element. This would be represented in the EMSA as a specific complex. Accordingly, EMSAs were performed with SVT2 WCE and the PRDII regulatory element as the radiolabeled DNA probe. The addition of PKR-GST to the EMSA binding reaction induced a specific complex (which most likely represents active NF-κB binding to the PRDII element). The NF-κB complex was induced with increasing amounts of PKR-GST (Fig. 2, lanes 10–12). This complex was also induced with mAb-purified mammalian dsRNA-activated PKR from Daudi cells (data not shown). The specificity of PKR-GST-induced complex formation was tested by using the p43 v-abl tyrosine kinase and the [Arg^{296}]PKR-GST mutant, which is not able to autophosphorylate (4). Neither the p43 v-abl tyrosine kinase nor the mutant PKR-GST was able to induce the NF-κB complex (Fig. 2, lanes 4–9), indicating that the complex was specifically induced by PKR-GST.

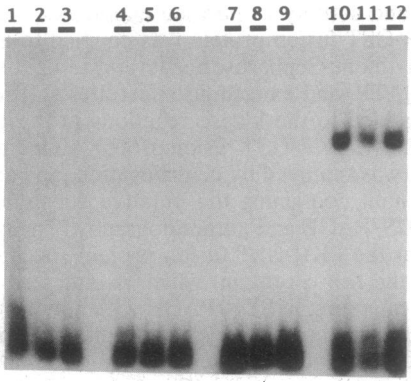

FIG. 2. *In vitro* activation of NF-κB by PKR-GST. SVT2 extract (1 μg) was preincubated with the following kinases: PKR-GST, [Arg^{296}]PKR-GST mutant, or p43 v-abl protein kinase for 10 min at RT, and end-labeled PRDII probe was added to the reactions for 15 min at RT. Lanes: 1–9, a series of negative controls including a binding reaction that was not treated with the PKR-GST (lane 1), glutathione-Sepharose bead treatment (lane 2), GST attached to the glutathione-Sepharose beads (lane 3), increasing amounts of the tyrosine kinase (5, 10, and 50 ng in lanes 4–6), and increasing amounts of [Arg^{296}]PKR-GST mutant (5, 10, and 50 ng in lanes 7–9); 10–12, PKR-GST treatment with 10, 5, and 50 ng of protein, respectively.

To determine if the NF-κB complex was specific for the PRDII element, oligonucleotide competitions were performed. The NF-κB complex was partially blocked by competition with a 5-fold excess of nonradioactive PRDII probe and was almost completely blocked by competition with a 25-fold excess (Fig. 3 *Left*, lanes 3–5). However, a 25-fold excess of a nonspecific oligonucleotide ISRE showed little competition, indicating that the putative NF-κB complex was specific for the PRDII element (Fig. 3 *Left*, lanes 6–8).

The identity of the putative NF-κB complex was confirmed by using EMSA in conjunction with antibody mobility shifts. After induction of the complex with PKR-GST, p50 antibody was added to the EMSA binding reaction. The p50 antibody clearly altered the mobility of the putative NF-κB complex (Fig. 3 *Right*, lane 3). This altered mobility was not due to the binding of the p50 antibody to the PRDII probe because a complex was not formed when the p50 antibody was added to the SVT2 extract in the absence of PKR-GST (Fig. 3 *Right*, lane 4). Thus, the complex induced by PKR-GST is a form of NF-κB containing the p50 subunit.

The phosphorylation state of IκB-α is crucial to NF-κB activation by PKR. This is clearly demonstrated by the addition of the unphosphorylated versus the phosphorylated forms of IκB-α to PKR-GST-activated SVT2 extracts (Fig. 4). In this experiment the NF-κB was activated by PKR-GST, which was subsequently removed by centrifugation. Increasing amounts of IκB-α protein were added to these binding reactions, and its

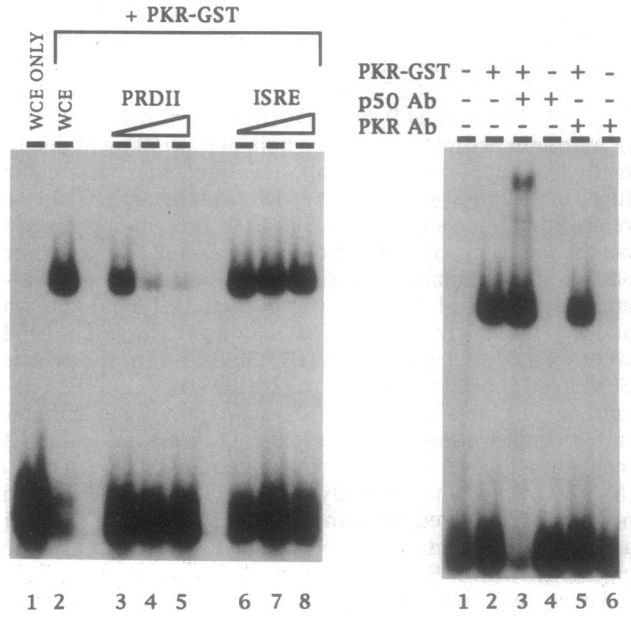

FIG. 3. Identification of the PKR-GST-induced complex as NF-κB. (*Left*) The NF-κB complex was activated in SVT2 WCE extracts with the addition of 50 ng of PKR-GST (lanes 2–8), and the unlabeled DNA oligonucleotide competitors used were the ISRE and PRDII elements. Lanes: 1, negative control (1 μg of SVT2 WCE extract with the end-labeled PRDII probe without the addition of PKR-GST); 2, positive control (same as lane 1 with PKR-GST); 3–5, addition of the specific DNA oligonucleotide competitor PRDII element at 5-, 10-, and 25-fold excess; 6–8, addition of the nonspecific DNA oligonucleotide competitor ISRE at 5-, 10-, and 25-fold excess. (*Right*) Effect of p50 antibody on NF-κB complex mobility. NF-κB was activated in the SVT2 extracts with the addition of 50 ng of PKR-GST (lanes 2, 3, and 5) and then either p50 antibody or PKR antibody was added to the EMSA reaction. Lanes: 1, negative control with SVT2 extract only; 2, addition of PKR-GST to SVT2 extract, activating NF-κB; 3, addition of p50 antibody to NF-κB-activated SVT2 extract; 4–6, negative controls—namely, addition of p50 antibody to nonactivated NF-κB SVT2 extract (lane 4), addition of PKR antibody to activated NF-κB SVT2 extract (lane 5), and addition of PKR antibody to nonactivated NF-κB SVT2 extract (lane 6).

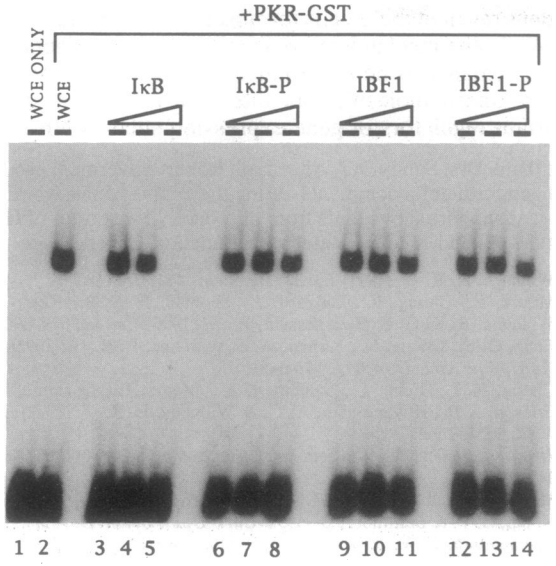

FIG. 4. The different phosphorylation states of IκB-α determine its ability to inactivate NF-κB. EMSA were performed as in Fig. 3, except that the binding reactions containing activated NF-κB were pretreated with various types and amounts of recombinant proteins. NF-κB was activated with the addition of PKR-GST, which was subsequently removed by centrifugation. The phosphorylated or unphosphorylated form of IκB-α was then added to the reactions, followed by the PRDII probe. Lanes: 1, negative control (SVT2 extract and PRDII probe only); 2–14, treatment with 50 ng of PKR-GST. Nonphosphorylated IκB-α was added at 5, 10, and 25 ng (lanes 3–5, respectively); phosphorylated IκB-α was added at 25, 50, and 100 ng (lanes 6–8); IBF-1 was added at 25, 50, and 100 ng (lanes 9–11); and IBF-1 that was preincubated with PKR-GST and cold ATP was added at 25, 50, and 100 ng (lanes 12–14).

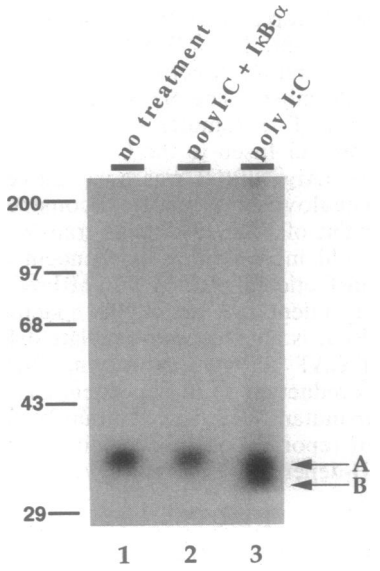

FIG. 5. *In vivo* dsRNA-dependent phosphorylation of IκB-α (MAD-3). Raw 264.7 cells were labeled with [^{32}P]orthophosphate and either treated (lanes 2 and 3) or not treated (lane 1) with dsRNA. IκB-α was immunoprecipitated with IκB-α antibody. The specificity of binding of IκB-α antibody was determined by adding 1 μg of recombinant IκB-α protein to the immunoprecipitation reaction (lane 2) and analyzing by SDS/PAGE.

effect on NF-κB–DNA binding activity was determined by EMSA. NF-κB was partially inactivated by pretreating the EMSA binding reaction with 10 ng of IκB-α and was completely inactivated by the addition of 25 ng of IκB-α protein (Fig. 4, lanes 3–5). However, the addition of 100 ng of PKR-GST-phosphorylated IκB-α had little effect on NF-κB–PRDII complex formation (Fig. 4, lanes 6–8). NF-κB was specifically inactivated by IκB-α because a nonspecific recombinant protein, IBF-1 (J. Haque, unpublished data), had no effect on NF-κB DNA binding activity (Fig. 4, lanes 9–14). Thus, the unphosphorylated form of IκB-α protein was able to bind to and inactivate the NF-κB protein present in the SVT2 WCE. However, the phosphorylated form of IκB-α was not able to bind to and inactivate NF-κB. These results are consistent with the model in which PKR phosphorylates IκB-α, and this phosphorylation event releases an active form of NF-κB.

To determine if dsRNA-dependent phosphorylation of IκB-α occurs *in vivo*, phosphorylation assays were performed. Mouse macrophage Raw 264.7 cells were incubated in phosphate-free medium with 0.1% FCS for 4 hr. The medium was removed, medium containing [^{32}P]orthophosphate with or without poly(rI·rC) was added, and the incubation was continued for an additional 4 hr. The cells were lysed, and IκB-α was immunoprecipitated with IκB-α antibody and subsequently analyzed by SDS/PAGE. The results indicate that dsRNA induces the formation of an unusual phosphoprotein (Fig. 5, lane 3). To confirm the identity of this phosphoprotein (termed "B"), unphosphorylated bacterially produced IκB-α protein was added to the immunobinding reaction. Unphosphorylated IκB-α protein competitively blocked phosphoprotein B, identifying this band as IκB-α (Fig. 5, lane 2). Thus, dsRNA treatment of Raw 264.7 cells induces the phosphorylation of IκB-α consistent with *in vitro* evidence indicating that PKR regulates NF-κB by phosphorylating IκB-α. An additional phosphoprotein (termed "A") was present in untreated and dsRNA-treated cells (Fig. 5, lanes 1–3). Phosphoprotein A could represent nonspecific binding to IκB-α antibody, since it was not competitively blocked by unphosphorylated IκB-α protein (Fig. 5, lane 2). Alternatively, phosphoprotein A could represent a constitutively phosphorylated form of IκB (24) that has an increased affinity for IκB-α antibody and therefore cannot be competitively blocked by unphosphorylated IκB-α protein.

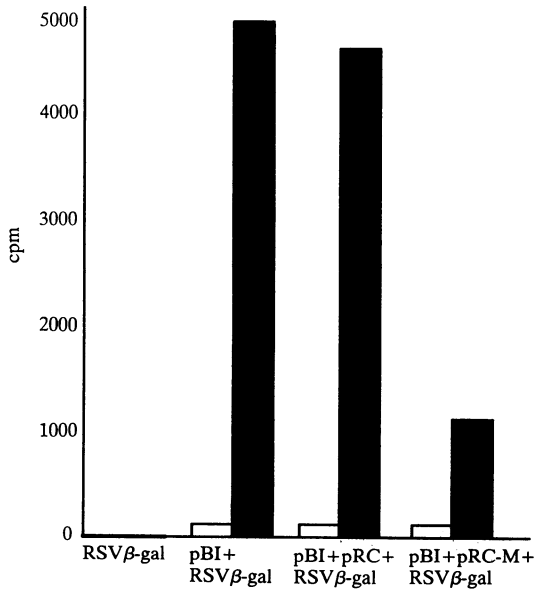

FIG. 6. Mutant PKR down-regulates dsRNA-dependent κB-CAT activity *in vivo*. Raw 264.7 cells were transiently transfected with the following: RSVβ-gal, pBI + RSVβ-gal, pBI + pRC vector + RSVβ-gal, and pBI + pRC-M + RSVβ-gal; each subsequently was induced with poly(I·C) at 100 μg/mL for 8 hr. ■, With poly(I·C) addition; □, without poly(I·C).

To show that PKR regulates NF-κB *in vivo*, a catalytically inactive PKR mutant, [Arg^{296}]PKR, was transfected into Raw 264.7 cells, and its effect on NF-κB-dependent CAT activity was determined. The NF-κB enhancer site derived from the human TNF-α promoter was cloned upstream of the minimal *tk* promoter fused to the CAT gene (19) (reporter construct pBI). [Arg^{296}]PKR was constitutively expressed under cytomegalovirus promoter (construct pRC-M). dsRNA treatment of Raw 264.7 cells transfected with pBI induced a 20-fold increase in κB-dependent CAT activity (Fig. 6). Cotransfection of pRC-M with pBI reduced dsRNA-induced κB-dependent CAT activity by a factor of 5. Therefore, mutant PKR is able to down-regulate dsRNA-induced κB-dependent CAT activity, implicating PKR in dsRNA signaling. This reduction in κB-dependent CAT activity can be attributed to mutant PKR, since cotransfection of the pRC vector and pBI reporter construct resulted in no significant decrease in κB-dependent CAT activity.

DISCUSSION

We have clearly demonstrated that PKR can regulate NF-κB. We show bacterially produced PKR directly phosphorylates IκB-α (MAD-3), thereby activating NF-κB–DNA binding activity *in vitro*. dsRNA also induces an unusual phosphorylated form of IκB *in vivo*. Extracts from mouse macrophage Raw 264.7 cells treated with dsRNA and immunoprecipitated with anti-IκB-α revealed a dsRNA-dependent alternative phosphorylated form of IκB-α. The identity of this band as IκB-α was confirmed by competitively blocking the immune precipitation reaction with the unphosphorylated form of IκB-α. It is likely that IκB-α is constitutively phosphorylated in Raw 264.7 cells, but treatment with dsRNA alters the phosphorylation status, perhaps rendering IκB-α more susceptible to regulation by proteolytic activity.

We demonstrated that PKR regulates NF-κB *in vivo* by transfecting a catalytically inactive PKR mutant into Raw 264.7 cells and measuring its effect on κB-dependent CAT activity, which increases 20-fold with dsRNA treatment. The mechanism by which [Arg^{296}]PKR inhibited this increase is not clear. The mutant could act in a transdominant mode heterodimerizing with the endogenous PKR (25). Alternatively, since [Arg^{296}]PKR can still bind dsRNA, it may act by sequestering this inducer (2, 3).

The regulation of transcription factor NF-κB has proven to be complex. This complexity is due in large part to the number and variety of different ways in which different subunits of NF-κB and IκB can interact. Two mechanisms have been implicated in this regulation, including proteolysis of NF-κB precursors and phosphorylation of NF-κB inhibitors. Substantial *in vitro* and *in vivo* evidence has been reported for both forms of regulation (26–35). It is conceivable that the proteolysis and phosphorylation regulatory pathways are distinct from each other. Alternatively, NF-κB is activated by phosphorylation and release of its inhibitor (IκB or IκB-like proteins), which are then degraded by specific protease(s).

Transcriptional activation of the IFN-β promoter by dsRNA requires the assembly and interaction of a number of factors. For example, in response to dsRNA, IRF-1 is activated and binds its cognate DNA elements PRDI and PRDIII of the IFN-β gene promoter (36). However, when mouse L929 cells were treated with the Ser/Thr-protein kinase inhibitor staurosporin and dsRNA, a *tk* promoter-CAT gene construct regulated by the IRF-1 binding-site hexamer was not induced, implicating a Ser/Thr-protein kinase in the modification of IRF-1 (37). Whether PKR can modify the activity of any of these factors required for IFN induction should be determined.

Recently it has been demonstrated that mutants of PKR, including the one used in this study, when expressed in NIH 3T3 cells result in these cells forming tumors in nude mice (38, 39). While the mechanism involved in this tumorigenic phenotype remains to be determined, our data demonstrate a mutant kinase molecule can alter the activation state of important regulators of gene expression such as NF-κB.

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