Nuclear Factor 90 Is a Substrate and Regulator of the Eukaryotic Initiation Factor 2 Kinase Double-stranded RNA-activated Protein Kinase

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Nuclear factor 90 (NF90) is a member of an expanding family of double-stranded (ds) RNA-binding proteins thought to be involved in gene expression. Originally identified in complex with nuclear factor 45 (NF45) as a sequence-specific DNA-binding protein, NF90 contains two double stranded RNA-binding motifs (dsRBMs) and interacts with highly structured RNAs as well as the dsRNA-activated protein kinase, PKR. In this report, we characterize the biochemical interactions between these two dsRBM containing proteins. NF90 binds to PKR through two independent mechanisms: an RNA-independent interaction occurs between the N terminus of NF90 and the C-terminal region of PKR, and an RNA-dependent interaction is mediated by the dsRBMs of the two proteins. Co-immunoprecipitation analysis demonstrates that NF90, NF45, and PKR form a complex in both nuclear and cytosolic extracts, and both proteins serve as substrates for PKR in vitro. NF90 is phosphorylated by PKR in its RNA-binding domain, and this reaction is partially blocked by the NF90 N-terminal region. The C-terminal region also inhibits PKR function, probably through competitive binding to dsRNA. A model for NF90-PKR interactions is proposed.

Nuclear factor 90 (NF90) is a cellular protein initially purified in complex with nuclear factor 45 (NF45) through its ability to bind to the antigen response recognition element in the interleukin-2 promoter (1). Independently, our laboratory identified NF90 as a cellular protein that interacts with VA RNA1, a structured adenoviral RNA, and with double-stranded RNA (dsRNA) (2). NF90 is one of the most abundant dsRNA-binding proteins in human cells, interacting preferentially with highly structured RNAs, in the order dsRNA > VA RNA1 > VA RNA2 > single stranded RNA (ssRNA). NF90 and NF45 have also been purified from human placental extracts in association with the protein synthesis eukaryotic initiation factor 2 and the catalytic subunit of the DNA-activated protein kinase DNA-PK (3). NF90 stabilizes the interactions between the catalytic subunit of DNA-PK and its regulatory heterodimeric DNA-binding subunits, Ku, in vitro, and it serves as a substrate for the activated DNA-PK in vitro.

NF90 contains no recognized DNA binding motifs; rather it contains two dsRNA-binding motifs (dsRBMs) which lie downstream of a bipartite nuclear localization signal. Several homologues of NF90 have since been identified, including Xenopus 4F.1 (4), Spnr (5), and ILF3 (6) in mouse, and p74 in rat (7). All of these proteins share homology at their N terminus to a mouse protein, Zfr, a protein of unknown function which has a conserved homologue in Drosophila (8) (Fig. 1A). Downstream of this Zfr homology domain is a region of homology which these proteins share with NF45, which we term the NF45 homology domain.

Several NF90 homologues and variants have been identified in human cells, and recent work from Duchange and colleagues (9) has helped clarify the relationships of several of these proteins. Alternatively spliced variants of the protein exist, including the human dsRNA-binding protein DRBP76, isolated during a two-hybrid screen for proteins that interact with PKR (10); translational control protein 80, which has been implicated in translational control (11); and M phase phosphoprotein 4, which is phosphorylated during the mitotic phase of the cell cycle (12). Most of these proteins differ as a result of two alternative splicing events, one at exon 14 which results in the addition of four additional amino acids between the two dsRBMs, and another at exon 17 which alters the C terminus of the protein downstream of the two dsRBMs (9). NF90 seems to be a variant of the ILF3 gene, as they are nearly identical at the nucleotide level although they diverge after the second dsRBM, due to a 2-nucleotide insertion in the NF90 sequence.

Proteins in this family have been implicated in the regulation of gene expression. Translational control protein 80 is reported to mediate the translational regulation of acid β-glucosidase, the enzyme deficient in Gaucher disease (11, 13), while the Xenopus homologue of NF90 was identified as part of CCAAT box transcription factor, a developmentally regulated transcription factor complex (14). Corthesy and Kao (1) showed that antisera to NF90 and NF45 reduce transcription from a minimal promoter containing three copies of the antigen response recognition element-2 element in vitro (1), and recent work from our laboratory has demonstrated that NF90 can serve as both a positive and negative regulator of transcription in human cells.2

NF90 and some of its homologues have been shown to interact with PKR (7), and serve as a substrate for this RNA-dependent protein kinase (10, 15). PKR also contains two

2 T. W. Reichman, L. C. Muniz, and M. B. Mathews, submitted for publication.
dsRBMs and binds preferentially to duplex and structured RNAs including the VA RNAs. Activation of PKR by dsRNA leads to the phosphorylation of translation initiation factor eIF2 on its α subunit, resulting in the general inhibition of protein synthesis. PKR is inhibited by binding to VA RNA or, more weakly, VA RNA2 (16). PKR activation is an aspect of the interferon-induced host antiviral mechanism (16). In addition, PKR has been implicated in other cellular processes including apoptosis (17), cellular transformation (18), differentiation (19), splicing (20), and transcription (21). PKR has been implicated in other cellular processes including apoptosis (17), cellular transformation (18), differentiation (19), splicing (20), and transcription (21).

**MATERIALS AND METHODS**

**Plasmids**—Plasmids used for in vitro transcription-translation were pcDNA3-DAI (37), pSRG2Δl, pSRG2Δa, and pSRG2Δ4 (38). pTTT-luciferase (Promega), and pBSIIKS-NF90.3 obtained from P. Kao (36). A series of 3′ deleted versions was prepared from pBSIIKS-NF90.3 by digesting with HindIII, Apal, EcoRV, and SalI and religating the 5′ portion of the NF90 sequence with the vector to give the derivatives 1–591, 1–419, 1–314, and 1–150, respectively.

To construct pGST-NF90, an EcoRI-Smal fragment from pBSIIKS-NF90, which contains the NF90 cDNA, was inserted into EcoRI- and Smad-digested pGEX 4T-2 (Amersham Pharmacia Biotech), creating pGEX4T-2-NF90. An EcoRI-SalI fragment containing the 5′ end of NF90 was generated by polymerase chain reaction using primers nNF90 (5′-GAATTCCTGGCCCTACACGGTCCAATGCGAATTTTTGTGAATGA-3′) and rNF90 (5′-GAATTCTCTGTGGACGATGCTGGCATT-3′) with EcoRI and SalI sites shown in italics and the translational start site shown in boldface, and inserted between the EcoRI and SalI sites of pBSIIKS, creating p3′NF90. A SalI-SalI fragment from pGEX4T-2-NF90 containing the 3′ end of NF90 together with an EcoRI-SalI fragment from p3′NF90 were inserted into EcoRI- and SalI-digested pGEX 4T-2, yielding pGST-NF90. GST-90C, containing the C-terminal amino acids 334–671 was constructed by digesting GST-NF90 with EcoRI and AciI, filling in with T4 polymerase (New England Biolabs) and religating the PKR was obtained from B. R. Williams (Cleveland Clinic Foundation).

Plasmid pRSETa-NF90 was constructed by inserting the EcoRI-Smal fragment of NF90 from pGST-NF90 into the EcoRI-HindIII site of pRSETa (Invitrogen, Life Technologies), destroying the HindIII site in the polyclinker. The C-terminal truncations pRSETa NF90 1–591, pRSETa NF90 1–334, and pRSETa NF90 1–150 were constructed by linearity of full-length pRSETa-NF90 with HindIII (1–591), AciI (1–334), or SalI (1–150), filling in with Klenow fragment (New England Biolabs), and adding a SpeI-TTL linker (New England Biolabs). The plasmids, pRSETa-NF90 334–671 and pRSETa-NF90 334–591 were constructed by digesting pRSETa-NF90 or pRSETa-NF90 1–591, respectively, with EcoRI and AciI, filling in with T4 DNA polymerase, and religating the plasmids. The plasmids, pRSETa-476–671 and

![FIG. 1. Wild-type NF90 protein and deletion constructs.](http://www.jbc.org/)

A, schematic of full-length NF90 showing its homology with NF45 and the ZF family of proteins as well as its dsRBMs. B, schematic of NF90 deletions used in this report. Amino acid numbers are indicated. C, Coomassie Brilliant Blue-stained gel of full-length and truncated histidine-tagged NF90, purified under denaturing conditions using metal affinity chromatography, compared with full-length NF90 additionally purified by poly(ethylene glycol) chromatography (lane 1). Molecular masses of protein standards are marked in kDa.
RNA Synthesis and Binding Assays— ssRNA and dsRNA was synthesized in vitro using pBS II KS+ (Stratagene, Inc., La Jolla, CA) and purified as described (39). Nitrocellulose filter binding assays were conducted essentially as described (39). Briefly, labeled RNA was incubated for 30 min at room temperature with bacterially purified recombinant protein in a reaction volume of 20 μl in binding reaction buffer (15 mM HEPES, pH 7.4, 5 mM MgCl$_2$, 1 mM DTT, 1 μM phenylmethylsulfonyl fluoride, 1 μg of aproptinin, leupeptin, and pepstatin/ml, 0.1 mg/ml bovine serum albumin, 0.1 mg/ml yeast total RNA). After dilution with 10 volumes of wash buffer (20 mM HEPES, pH 7.4, 50 mM KCl, 1.5 mM MgCl$_2$, 0.1 mM EDTA), the reaction mixtures were immediately filtered in a slot-blot apparatus through a 0.45-μM pore size nitrocellulose membrane (Schleicher and Schuell) presoaked for 20 min at room temperature. Each well was washed two times with 250 μl of ice cold wash buffer, and the filter was dried and exposed to a Packard Instant Imager for quantitation.

In Vitro Transcription and Translation—Proteins were synthesized and labeled in vitro in the presence of [35S]methionine (ICN) using the TNT T7 coupled wheat germ system (Promega) according to manufacturers instructions.

Co-immunoprecipitation Analysis—Polyclonal antibody against NF90 was obtained from P. Kao (36). Anti-PKR polyclonal antibody was described previously (38). Wheat germ translation products (10–20 μl) were treated with RNase A at 300 μg/ml for 1 h at 30 °C, mixed with other wheat germ translation products where indicated, then incubated at 4 °C in the presence of 5–10 μl of antibody for 2 h with rocking. Immune complexes were collected using protein A-Sepharose beads (Amersham Pharmacia Biotech) and washed 5 times with 500 μl of buffer A (20 mM HEPES, pH 7.4, 50 mM KCl, 0.1% Nonidet P-40, 0.5 mM DTT). The beads were resuspended in 30 μl of 2 × Laemmli sample buffer (40), boiled for 3 min, resolved by SDS-polyacrylamide gel electrophoresis, and visualized by autoradiography.

GST Pull-down Assays—Plasmids encoding GST, GST-NF90, GST-NF90334–671, or GST-PKR were used to transform Escherichia coli BL21 (DE3) strain. Fusion protein expression was induced with 1 mM isopropyl-1-thio-D-galactopyranoside for 3 h at 37 °C and bacterial pellets were frozen at −80 °C. Pellets were subsequently resuspended in 30 μl of 2 × Laemmli sample buffer (40), boiled for 3 min, resolved by SDS-polyacrylamide gel electrophoresis, and visualized by autoradiography.

FIG. 2. NF90 binds to dsRNA and ssRNA. A, results of a filter binding assay using 320 ng (5 nM) of full-length NF90 with increasing concentrations of 85-nucleotide dsRNA and ssRNA synthesized in vitro. Data are the mean of triplicate assays. Similar results were obtained in two experiments and with dsRNA and ssRNA 104 nucleotides in length. B, binding of 5 nM full-length or truncated NF90 protein (amino acid numbers indicated) to 3.4 nM ssRNA (white bars) or dsRNA (gray bars). Filter binding assays were conducted 3 times in triplicate. C, binding of wild-type and NF90 and its deleted derivatives, as in B except that the RNA concentration was 32 nM.
NF90 binds to both ssRNA and dsRNA via its dsRBMs—The NF90 sequence predicts two dsRBMs in the proteins C-terminal half. Previous reports indicated that NF90 and some of its family members and homologues interact with dsRNA (2, 4, 7, 10, 15). To determine whether the two dsRBMs are involved in RNA binding, we constructed a panel of N- and C-terminal truncations. PKR, NF90 DERivatives containing amino acids 1–314, 1–416, 1–314, and 1–150, and luciferase (Lue) were synthesized in wheat germ extracts (Fig. 2A) and analyzed in nitrocellulose filter binding assays using dsRNA (2, 4, 7, 10, 15). After renaturation, the RNA binding activity was equimolar amounts (5 nM) of full-length dsRNA and ssRNA, 85 bases in length, synthesized in wheat germ extracts as unlabeled (Cold Protein) or radiolabeled (35S-Protein) proteins as indicated. Extracts containing radiolabeled protein, or mixtures of radiolabeled and unlabeled proteins, were subjected to immunoprecipitation with antibodies to PKR (lanes 1–4) or NF90 (lanes 5–8), and analysis by polyacrylamide gel electrophoresis and autoradiography. Arrows mark the positions of PKR and NF90. B, cytosolic (left panel) and nuclear (right panel) extracts of 293 cells were immunoprecipitated with antibodies to PKR (lanes 2 and 7), NF90 (lanes 3 and 8), NF45 (lanes 4 and 9), or actin (lanes 5 and 10), or with preimmune serum (lanes 6 and 11). The immunoprecipitates, together with 293 extract to provide markers for NF90 and NF45 (lane 1), were separated by SDS-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membranes, and then probed with antibodies to NF90 and NF45.

RESULTS

NF90 Binds to Both ssRNA and dsRNA via Its dsRBMs—The NF90 sequence predicts two dsRBMs in the proteins C-terminal half. Previous reports indicated that NF90 and some of its family members and homologues interact with dsRNA (2, 4, 7, 10, 15). To determine whether the two dsRBMs are involved in RNA binding, we constructed a panel of N- and C-terminal truncations (Fig. 1B), and expressed the recombinant proteins in bacteria. These proteins carry a polyhistidine tag at their N termini, allowing their purification under denaturing conditions using a metal affinity resin which binds to the His-tag (Fig. 1C). After renaturation, the RNA binding properties of full-length and truncated forms of NF90 were analyzed in nitrocellulose filter binding assays using dsRNA and ssRNA, 85 bases in length, synthesized in vitro (39).

Initially we assessed the ability of full-length NF90 to bind to dsRNA and ssRNA over a broad range of RNA concentrations. Although NF90 binds dsRNA preferentially at low RNA concentrations, at higher concentrations (32 nM) it binds both dsRNA and ssRNA with similar efficiency (Fig. 2A). To map the RNA binding activities, equimolar amounts (5 nM) of full-length dsRNA and ssRNA with similar efficiency (Fig. 2B). Thereafter, we focused on high RNA concentrations. These data indicate that the ssRNA and dsRNA binding activities reside in the domain of NF90 which...
NF90 Interacts with PKR

Interactions between some NF90 family members and PKR have been observed (7, 10, 15) but are responsible for NF90 binding to both dsRNA and ssRNA.

NF90 Interacts with PKR—Interactions between some NF90 family members and PKR have been observed (7, 10, 15) but these reports did not distinguish between direct protein-protein interactions and those mediated by RNA. We used co-immunoprecipitation assays to define the interactions between NF90 and PKR and to determine the contribution, if any, of RNA binding to these interactions.

First, NF90 and PKR were synthesized by coupled transcription and translation in wheat germ extracts. In some cases \(^{35}S\)methionine was included in the reaction to label the protein being synthesized. Fig. 3A shows that PKR and NF90 labeled in this way were immunoprecipitated by their corresponding antibodies (lanes 1 and 5, respectively) but, as expected, neither protein was immunoprecipitated by the heterologous antiserum (lanes 2 and 6). In the presence of unlabeled PKR (lane 3), labeled NF90 was co-immunoprecipitated by antibody to PKR, consistent with the formation of a PKR-NF90 complex when wheat germ extracts containing the two proteins are mixed. The control protein luciferase did not co-immunoprecipitate under the same conditions (lane 4). Reciprocally, anti-NF90 antiserum co-immunoprecipitated PKR but not luciferase in the presence of unlabeled NF90 (lanes 7 and 8). As further tests of specificity, neither anti-PKR nor anti-NF90 antisera immunoprecipitated luciferase synthesized \textit{in vitro}, and preimmune serum did not immunoprecipitate PKR or NF90 either singly or from mixed translation extracts (data not shown). This \(^{35}S\)-coIP experiment demonstrates that PKR and NF90 interact \textit{in vitro}. Similar data verify the direct interaction of NF90 and NF45 (data not shown).

To test for interactions between NF90, NF45, and PKR \textit{in vivo}, we performed co-immunoprecipitation experiments from cytosolic and nuclear extracts of 293 cells using antibodies directed against each of the three proteins. Anti-actin and preimmune serum were included as negative controls. The immunoprecipitates were resolved by gel electrophoresis, transferred to a nitrocellulose membrane, and probed with antibody directed against NF90 or NF45. These “IP-Western” assays revealed that NF90 and NF45 were immunoprecipitated with cognate antibody, and co-immunoprecipitated with one another, as expected (Fig. 3B, lanes 3, 4, 8, and 9), but not with preimmune serum or antibody directed against actin (lanes 5, 6, 10, and 11). Additionally, NF90 and NF45 were co-immunoprecipitated with antibodies directed against PKR (lanes 2 and 7). Since NF45 does not directly interact with PKR \textit{in vitro},\(^3\) we conclude that PKR is found in complexes with NF90 and NF45 in both cytosolic and nuclear extracts.

PKR-NF90 Interaction Sites—These co-immunoprecipitation assays were conducted in the presence of RNase and high salt to reduce the likelihood that the interactions were bridged by RNA. To determine explicitly whether RNA binding or the RNA-binding domain of NF90 is involved in its interactions with PKR, we examined a series of truncated forms of NF90 for their ability to bind PKR using the \(^{35}S\)-coIP procedure described above.

Truncated forms of NF90 were labeled by coupled transcription and translation in wheat germ extracts. They were all immunoprecipitated by antibody against NF90 (Fig. 4A, lanes 1, 4, 8, and 11), but labeled luciferase was not (data not shown). All of the \(^{35}S\)-labeled NF90 truncations were co-immunoprecipitated by antibody directed against PKR when the labeled protein was mixed with wheat germ extract containing unlabeled \textit{in vitro} synthesized PKR (lanes 2, 5, 9, and 12), but not in the absence of PKR (lanes 3, 6, 10, and 13). The luciferase control was not co-immunoprecipitated (lane 7). These data demonstrate that the interaction of NF90 with PKR occurs in the absence of NF90s RNA-binding domain and requires only the first 150 amino acids of the protein. The resistance of the complexes to RNase digestion and washing with relatively high concentrations of salt (750 mM) are suggestive of direct protein-protein interactions.

To extend these results we used a “GST pull-down” assay. Both N-terminal and C-terminal deletions of NF90, synthesized \textit{in vitro} by transcription and translation in wheat germ extracts, were mixed with GST or GST-PKR bound to glutathione beads. GST-PKR bound to full-length NF90 but not luciferase (Fig. 4B, compare lanes 3 and 4, and quantitation in Fig. 4C). As expected, the shortest N-terminal NF90 fragment (amino acids 1–150; lane 7) interacted with GST-PKR, albeit to a lesser extent than full-length NF90. Truncated forms of NF90 containing both dsRBMs were also able to interact with GST-PKR; the minimal requirement defined by these data was amino acids 334–591 of NF90 (lanes 5, 8, 9, and 13). A truncation containing the first dsRBM alone (amino acids 150–419; lane 12) interacted with GST-PKR but to a lesser extent than

\(^3\) L. M. Parker and M. B. Mathews, unpublished observations.
when both domains were present, while those containing only the second dsRBM (truncated at amino acids 476–671) failed to bind to GST-PKR (lanes 10 and 11). These results suggest that the first dsRBM contributes more to the interaction of these two proteins than the second although both are needed for optimal binding. From this experiment, as well as that of Fig. 4A, we conclude that NF90 contains two separate domains capable of interacting with PKR, an N-terminal domain consisting of the first 150 amino acids of NF90 and a C-terminal domain which overlaps the dsRBMs.

Unexpectedly, the deleted form of NF90 containing amino acids 1–334 failed to interact with GST-PKR (Fig. 4B, lane 6), in contrast to the shorter form consisting of amino acids 1–150 (lane 7) and the results shown in Fig. 4A where two NF90 derivatives containing C-terminal truncations at amino acids 314 and 426 both interacted with PKR. It is possible that the removal of NF90 residues after amino acid 334 results in misfolding of the proteins N-terminal region, thereby preventing its interaction with PKR. Alternatively, truncation at amino acid 334 may reveal the existence of an inhibitory domain in the middle of NF90 (amino acids 150–334) that prevents the interaction of the N terminus of NF90 with PKR. While direct evidence on this issue is sparse, accumulating data suggest that the central portion of NF90 governs its ability to assume an active conformation.2

dsRBMs-dependent and -independent PKR-NF90 Interactions

The interaction of the NF90 N-terminal domain with PKR is presumably RNA-independent because this part of NF90 does not interact detectably with RNA (Fig. 2), but it is possible that RNA mediates the interactions between the C-terminal domain of NF90 and PKR. As a direct test, we examined PKR mutants impaired in dsRNA binding for their ability to interact with the GST-NF90 fusion protein. Wild-type PKR (wt) and its derivatives LS16 and H90041 were synthesized in vitro in the presence of [35S]methionine. PKR LS16 and PKR H90041 carry a point mutation and a deletion of dsRBM1, respectively,
abolishing their ability to bind dsRNA (38). All three forms of PKR interacted with the GST-NF90 fusion protein (Fig. 5A, lanes 4–6) but not with GST alone (lanes 7–9), confirming that the interaction between NF90 and PKR is not dsRNA-dependent. The binding of PKR to GST-NF90 was somewhat impaired by the RNA binding mutations of PKR (40–60%; compare lanes 5 and 6 to lane 4) suggesting that the dsRNA binding activity of PKR contributes to PKR-NF90 interactions.

We further addressed this point by examining PKR mutants for their ability to interact with a GST-NF90 deletion containing only amino acids 334–671. This GST-NF90 construct contains the RNA-binding region of NF90, but not its N-terminal 150 amino acids which also interact with PKR. Wild-type PKR and three mutants (LS16, Δ1, and Δ4) were synthesized in vitro in the presence of [35S]methionine. PKR Δ4 has a deletion outside the PKR RNA-binding domain (amino acids 481–524), and still binds to dsRNA (38). While full-length PKR and PKR Δ4 both interacted with the C-terminal half of NF90 in this assay (Fig. 5B, lanes 5 and 14), the two RNA-binding mutants of PKR (LS16 and Δ1) were unable to interact with the C-terminal half of NF90 (lanes 2, 8, and 11). These data confirm that there are two separate interactions between NF90 and PKR, an RNA-dependent interaction mediated by the dsRBMs of the two proteins and an RNA-independent interaction mediated by the N-terminal 150 amino acids of NF90. The observation that PKR Δ4 failed to bind as efficiently as full-length PKR suggests that the RNA-independent interaction may involve amino acids 481–524 of PKR.

**NF90 and NF45 Are Substrates for PKR in Vitro**—Since PKR interacts with NF90 and NF45, as shown above (Fig. 3B), it is possible that both NF90 and NF45 might serve as substrates for PKR. Previously, NF90 and its homologue DBP76 were identified as substrates for PKR (10, 15). To confirm this observation and determine whether NF45 can also serve as a PKR substrate, we tested their ability to be phosphorylated by PKR in an in vitro kinase assay. The activation and autophosphorylation of PKR is dependent on dsRNA (Fig. 6A, lanes 2 and 4). Both NF90 and NF45, present in a fraction from a poly(I)-poly(C) column fraction from 293 cells (2), were phosphorylated by PKR (lane 8), and this labeling was dependent on dsRNA and PKR (lanes 5–7). We further tested purified recombinant NF90 (Fig. 6B) and NF45 (Fig. 6C) to determine whether they also serve as substrates for PKR. Both bacterially expressed NF90 and NF45 were phosphorylated in a dsRNA-dependent and PKR-dependent fashion, indicating that they both serve as substrates for PKR in vitro.

Deletions were used to localize the region of NF90 that is phosphorylated by PKR. In this experiment, PKR was activated in the presence of dsRNA and unlabeled ATP before the addition of [γ-32P]ATP and NF90, so the PKR was only lightly labeled (Fig. 6D, arrow). As expected, PKR was activated only in the presence of dsRNA (lanes 1 and 2), and full-length recombinant NF90 was phosphorylated only in the presence of activated PKR (lanes 3 and 4). When activated PKR was incubated with equimolar amounts of the truncated NF90 proteins, the derivative lacking the NF90 C terminus (amino acids 1–591) was labeled approximately as efficiently as the full-length protein. The N-terminal NF90 fragments, 1–334 and 1–150, were not labeled to a detectable extent (lanes 6 and 7), whereas the C-terminal fragments 334–671 and 334–591 were heavily labeled (lanes 8 and 9). These results map the PKR phosphorylation sites on NF90 to the region containing its dsRBMs (amino acids 334–591), and indicate that the N-terminus of NF90 is partially inhibitory toward phosphorylation by PKR. This inhibition may reflect a conformational change in NF90 (see “Discussion”).

**NF90 Inhibits PKR Activation in Vitro**—Several RNA-binding proteins, including the cellular proteins TAR RNA-binding protein and PACT (25, 42) and viral proteins such as E3L (29, 43), are known to affect PKR activity. We tested full-length recombinant NF90 to determine whether it can modify the activity of PKR, by supplementing a kinase assay with increasing concentrations of recombinant NF90. The data in Fig. 7A show that 320 ng (5 nM) of added NF90 inhibited PKR kinase activity, and that 32 ng (0.5 nM) of NF90 gave partial inhibition (lanes 2 and 4–8).

To map this inhibitory activity to a particular region of NF90, equimolar amounts of several NF90 truncations were tested in a PKR kinase assay. No PKR was phosphorylated in the absence of dsRNA, regardless of the presence of NF90, indicating that recombinant NF90 has no activating effect on PKR in the absence of dsRNA (Fig. 7B, compare lanes 1 and 2 with lane 2). When dsRNA was present, 5 nM full-length NF90 inhibited PKR autophosphorylation (lane 3), as did those deleted proteins competent to bind RNA (lanes 5, 8, and 9). The two truncations lacking RNA binding activity were unable to inhibit PKR activity (lanes 6 and 7). The mapping of the inhibition to the dsRBM region raises two possible explanations for the inhibition: either the inhibitory effect of NF90 on PKR activity is due to interactions between the NF90 RNA-binding domain with PKR, preventing PKR dimerization and precluding its activation; alternatively, inhibition is due to competition between NF90 and PKR for the dsRNA required to activate PKR. To distinguish between these two possible explanations, we titrated increasing amounts of dsRNA into a PKR kinase assay. In the presence of 80 ng of NF90 (1.25 nM), partial inhibition of PKR activation was observed (Fig. 7C, compare lanes 4 and 5 with lanes 2 and 3). This inhibition was overcome by the addition of increasing amounts of dsRNA (lanes 6–9). Although high concentrations of dsRNA can prevent PKR activation (39), no inhibition of PKR activity was observed when the same amounts of dsRNA were added to a PKR kinase assay in the absence of NF90 (lanes 10–13). Since increasing
DISCUSSION

NF90 is part of an expanding family of highly conserved dsRNA-binding proteins. The vertebrate homologues of NF90 all contain dsRBMs, while a Drosophila homologue (8) contains zinc finger motifs, a motif involved in both RNA and DNA binding (44, 45). This report is the first characterization of the functional domains of NF90. Although initially identified based on its ability to bind to the antigen response recognition element DNA element (1), subsequent work demonstrated that NF90 interacts with structured RNA and poly(I-C)-agarose (2), as well as ssDNA- and dsDNA-cellulose (46). We have mapped the RNA-binding domain of NF90 to the region of the protein containing the dsRBMs, within the C terminus of the protein.

NF90 binds to both dsRNA and ssRNA through this domain at high RNA concentrations, although at lower concentrations it binds preferentially to dsRNA. While the physiological relevance of the interactions of NF90 with RNA remains to be elucidated, our data suggest that one function of NF90 may be to bind to structured ssRNA or dsRNA in order to regulate the function of PKR.

Work from other groups (7, 10, 15) has demonstrated that NF90 and some of its family members interact with PKR. We have characterized this interaction and mapped the domains of NF90 responsible for interacting with PKR. NF90 interacts with both the N and C termini of PKR through two mechanisms (Fig. 8, top). The N-terminal domain of NF90, composed of its first 150 amino acids, interacts with the C-terminal domain of PKR in an RNA-independent fashion; NF90 and PKR also interact in an RNA-dependent fashion via their RNA-binding domains. The latter interaction may also involve direct protein-protein interactions, as has previously been observed between PKR dimers (47), between PKR and E3L (29), and between PKR and PACT (31, 48).

NF90 is known to serve as a substrate for PKR in vitro (15) and this report presents data showing that NF45 also serves as a substrate for PKR. The domain of NF90 that is phosphorylated by PKR lies in the C terminus of the protein. The isolated C-terminal region of NF90, containing the two dsRBMs, is much more efficiently phosphorylated than the full-length protein, suggesting that the N-terminal region of NF90 impedes its phosphorylation by PKR. The N terminus of NF90, when added to a kinase assay alone, does not inhibit PKR function, consistent with a conformational change in NF90 allowing PKR increased access to the protein. As diagrammed in Fig. 8 (bottom), removal of interference by the N terminus of NF90 still allows the protein to interact with PKR through their dsRBMs and permits increased access to the sites of phosphorylation on NF90. Independent studies from our laboratory also imply that the N terminus of NF90 structurally precludes full activity of the protein. Reichman and co-workers (review) have suggested that NF45, which activates the function of NF90 in a transactivation assay, causes a conformational change in NF90 that increases its activity. It is interesting to speculate that NF45-induced conformational changes in NF90 also allow increased phosphorylation by PKR. Conversely, PKR phosphorylation of NF90 may serve the same function as NF45, altering the conformation of NF90 and allowing for increased activity.

We demonstrate that NF90 modulates the function of PKR, inhibiting its activity, probably through competitively binding to dsRNA. Once activated, however, NF90 does not inhibit PKR activity. Further work is needed to determine whether the inhibition of PKR by NF90 is simply through competitive binding to the dsRNA needed to activate PKR, as our data suggests, or whether it involves interactions between the proteins as well. These two methods of inhibition need not be mutually exclusive, as in the case of vaccinia virus E3L which inhibits PKR through competitive binding to dsRNA as well as through direct protein-protein interactions (29). Our model depicts the interaction as one between a monomer of NF90 and a monomer PKR; however, it is possible that NF90 interacts with PKR dimers, which may have further implications for its ability to inhibit PKR.

NF90 is predominantly nuclear, although it has been found in the cytoplasm by fractionation (2). PKR, in contrast, is predominantly cytoplasmic, although about 20% of cellular PKR has been observed in the nucleus by fluorescence microscopy, primarily localized to the nucleolus (35). Recent work has suggested that PKR may re-localize to the nucleus in response to DNA damaging agents (49), a connection that is underlined by the co-purification of NF90 with DNA-PK and Ku (3). It is possible that NF90 functions as an inhibitor of nuclear PKR, and this inhibition may be relieved through re-localization of PKR to the nucleus. The re-localized PKR may overwhelm the inhibition by NF90, or PKR could be re-localized to the nucleus in an active form which is able to phosphorylate NF90 as shown here, but is not inhibited by it.

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