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Functional Expression and RNA Binding Analysis of the Interferon-Induced, Double-Stranded RNA-Activated, 68,000-M_r Protein Kinase in a Cell-Free System

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Eukaryotic viruses have devised numerous strategies to downregulate activity of the interferon-induced, double-stranded (dsRNA)-activated protein kinase (referred to as p68 on the basis of its M_r of 68,000 in human cells). Viruses must exert this control to avoid extensive phosphorylation of the alpha subunit of eukaryotic initiation factor 2 (eIF-2) by p68 and the resultant negative effects on protein synthesis initiation. To begin to define the molecular mechanisms underlying this regulation, we optimized expression of p68 in an in vitro transcription-translation system utilizing the full-length cDNA clone. The in vitro-expressed kinase was autophosphorylated in response to dsRNAs and heparin in a manner similar to that for the native p68 provided that the kinase inhibitor, 2-aminopurine, was present during the in vitro translation reaction. Further, the activated kinase efficiently phosphorylated its natural substrate, the alpha subunit of eIF-2. Binding experiments revealed that the expressed kinase complexed with the dsRNA activator, reovirus dsRNA, as well as the adenovirus-encoded inhibitor, VAI RNA. Interestingly, both the reovirus RNAs and VAI RNA also complexed with protein kinase molecules that lacked the carboxyl terminus and all catalytic domains. Deletion analysis confirmed that the p68 amino terminus contained critical determinants for reovirus dsRNA and VAI RNA binding. Further, reovirus dsRNA efficiently bound to, but failed to activate, p68 kinase molecules containing a single amino acid substitution in the invariant lysine 295 present in catalytic domain II. Taken together, these data demonstrate that this expression system permits a detailed mutagenic analysis of the regions of p68 required for interaction with virus-encoded activators and repressors.

Both the p68 protein kinase and the 2′,5′-oligoadenylate synthetase are enzymes induced by interferon treatment and activated by double-stranded RNAs (dsRNAs) (for reviews, see references 16, 33, and 40). Activation of these enzymes has been suggested to play a role in the interferon response to virus infection, although definitive proof has not yet been obtained. The dsRNA-activated protein kinase (referred to as p68 on the basis of its M_r of 68,000) is a serine/threonine kinase which is characterized by two distinct kinase activities; the first involves an autophosphorylation (activation) reaction, and the second involves a protein kinase activity on exogenous substrates (11, 16). When activated, the p68 protein kinase phosphorylates its natural substrate, the alpha subunit of protein synthesis eukaryotic initiation factor 2 (eIF-2). Phosphorylation of the eIF-2 alpha subunit blocks the eIF-2B-mediated exchange of GDP for GTP required for catalytic utilization of eIF-2 (27, 39, 43). These events lead to limitations in functional eIF-2, which is essential for protein synthesis initiation and is required to bind initiator Met-tRNA to the ribosomal subunit before mRNA is bound (21).

It is now well documented that virus-specific RNAs synthesized during infection have the potential to activate p68 (3, 4, 26, 34, 41, 45). Thus, viruses must downregulate kinase activity or otherwise encounter declines in protein synthesis rates (4, 50). The strategies devised by eukaryotic viruses to block p68 kinase activity are numerous. For example, adenovirus encodes an RNA polymerase III product, VAI RNA, which complexes with and inactivates p68 (12, 24, 36). Poliovirus infection induces p68 degradation (4), while infection by another picornavirus, encephalomyocarditis virus, possibly causes p68 sequestration (7). Human immunodeficiency virus type 1 may mediate the necessary downregulation of p68 via action of the Tat regulatory protein (41, 42), whereas influenza virus blocks kinase activity by activating a cellular inhibitor of p68 (25, 26, 32). Finally, reovirus and vaccinia virus appear to downregulate the kinase by encoding gene products that bind to and sequester the dsRNA activator of p68 (1, 20, 52).

Despite these studies, little is known about the molecular mechanisms of activation and repression of the p68 protein kinase. We therefore optimized an in vitro transcription-translation system utilizing the recently cloned full-length cDNA encoding the human p68 kinase (38) to synthesize a functional p68 and perform a structure-function analysis of the in vitro-made protein kinase. In this first report, we have shown that the kinase inhibitor 2-aminopurine (2AP) must be present during the translation for optimal synthesis of a functional enzyme. Further, we determined that the invariant lysine 295 was essential for p68 kinase function since mutants lacking this amino acid completely lacked activity. Finally, we found that activator reovirus dsRNAs, and the adenovirus-encoded inhibitor VAI RNA, bound primarily within the amino terminus of the p68 kinase.

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MATERIALS AND METHODS

In vitro transcription of full-length and truncated p68 DNAs. The cDNA for p68 was subcloned in plasmid pcDNAI/NEO (Invitrogen) by using the *Hin*dIII and *Pst*I sites of p68. For transcription of full-length p68 mRNA (either wild type or domain II mutants), the recombinant p68-pcDNAI/NEO was linearized with *Eco*RV. Transcription from the T7 promoter in the presence of the cap analog 7-mGpppG was carried out essentially as described by Melton et al. (37) except that the final concentrations of ATP, CTP, UTP, and 7-mGpppG were raised to 4 mM and the final concentration of GTP was raised to 0.4 mM. Yields of transcripts were calculated from the incorporation of [α-^{32}P]UTP. A portion of each reaction was analyzed on an 8% polyacrylamide–7 M urea gel stained with ethidium bromide to confirm both the quantitation and the integrity of the in vitro-transcribed RNAs. Transcription of truncated messages was carried out exactly as described above except that the recombinant p68-pcDNAI/NEO was linearized at sites internal to the gene, resulting in shorter runoff transcripts (see Fig. 1).

Preparation of message-dependent rabbit reticulocyte lysate. Message-dependent rabbit reticulocyte lysate was prepared according to a protocol provided by Rosemary Jagus. Immature female New Zealand White rabbits were injected subcutaneously with 0.25 ml of a 2.5% phenylhydrazine chloride solution per kg of body weight on 5 consecutive days. The rabbits were allowed to rest for 2 days and were then bled by heart puncture, using ketamine hydrochloride as an anesthetic. The blood was centrifuged at 1,000 × *g* to pellet the reticulocytes, which were washed once in complete wash buffer (0.14 M NaCl, 5 mM KCl, 7.5 mM magnesium acetate, 1 mM glucose), centrifuged as before, washed once in wash buffer without magnesium acetate or glucose, and then centrifuged again at 1,000 × *g*. Two volumes of cold, RNase-free water was added to the final pellet and stirred on ice for 5 min. The solution was then centrifuged, and the supernatant was collected and snap-frozen in aliquots under liquid nitrogen. For preparation of mRNA-dependent lysate, the supernatant was treated with 150 U of *Staphylcoccus aureus* micrococcal nuclease per ml in the presence of 1 mM $CaCl_2$ and 5 U of hemin per ml. This mixture was incubated at 20°C for 20 min, and the reaction was terminated by the addition of EGTA to 2 mM. Finally, calf liver tRNA was added to a final concentration of 50 μg/ml.

In vitro translation in rabbit reticulocyte lysate. For the production of p68 proteins, the in vitro-transcribed mRNAs plus 2 μg of calf liver tRNA were added to the message-dependent lysate translation mix, which contained 50 mM KCl, 0.5 mM magnesium acetate, 150 μM amino acids (minus methionine, unless translation was done in the absence of [^{35}S]methionine), 18 μCi of [^{35}S]methionine, 15 mM phosphocreatine, 2 U of creatine phosphokinase per ml, 10 μM hemin, 262 U of RNAsin per ml, and message-dependent lysate comprising 73% of the final reaction volume. The reaction mixes were incubated at 30°C for 1-h, and the reaction was terminated by the addition of 2 mM NaOH and 100 μg of RNase A per ml. An equal volume of 2× protein electrophoresis buffer was added, and the products analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE).

Immunoprecipitation analysis. Immunoprecipitation of wild-type, mutant, and truncated p68 from in vitro translation reactions was carried out essentially as described previously (24). Briefly, after in vitro translations, the reaction mixtures were diluted in high-salt buffer I (20 mM Tris-HCl [pH 7.5], 50 mM KCl, 400 mM NaCl, 1 mM EDTA, 100 U of aprotinin per ml, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 1% Triton X-100, 20% glycerol). This mixture was then reacted for 2 h with the monoclonal antibody (MAb) to p68 (31). After incubation with the in vitro translation products, precipitates were washed four times with high-salt buffer I and three times with buffer II (10 mM Tris-HCl [pH 7.5], 100 mM KCl, 0.1 mM EDTA, 100 U of aprotinin per ml, 20% glycerol). Following the last buffer II wash, the immunoprecipitate was boiled in 2× protein electrophoresis disruption buffer and analyzed by SDS-PAGE. For experiments utilizing the polyclonal antibody, the in vitro translation mixtures were reacted with polyclonal p68 antibody (30), after which protein A-agarose was added.

Analysis of p68 autophosphorylation and activity in vitro. p68 RNA constructs were prepared, in vitro translated in the presence of unlabeled amino acids, and immunoprecipitated as described above. Following the last buffer II wash, the MAb-Sepharose-bound kinase complex was resuspended in kinase reaction buffer (20 mM Tris-HCl [pH 7.5], 0.01 mM EDTA, 50 mM KCl, 8 μg of aprotinin per ml, 0.3 mg of bovine serum albumin per ml, 2 mM $MgCl_2$, 2 mM $MnCl_2$, 1.25 μM [γ-^{32}P]ATP, 0.1 mM phenylmethylsulfonyl fluoride, 5% glycerol). The appropriate activators [poly(I-C)], reovirus dsRNAs, or heparin) were then added in various concentrations, and the mixtures were incubated at 30°C for 15 min. The reaction was stopped by adding 2× disruption buffer containing 50 mM EDTA and 20 μg of RNase A per ml. The reaction mixtures were boiled and analyzed by SDS-PAGE. For the activity assays, 0.5 μg of purified eIF-2 was added to the kinase reactions. The eIF-2 was purified by a modification of the procedure of Konieczny and Safer (27) to be published elsewhere.

Preparation of radiolabeled VAI RNA and reovirus dsRNAs. The VAI RNA (10, 47) was transcribed in vitro by using T7 RNA polymerase as described above except that the final concentrations of ATP, CTP, and GTP were 0.5 mM and that of UTP was 0.20 mM. The reaction mixtures included 50 μCi of [α-^{32}P]UTP. 5′ end labeling of reovirus dsRNAs of reovirus type 3 (generously provided by Aaron Shatkin) was carried out in the presence of [γ-^{32}P]ATP as described previously (2). Both the VAI RNA transcriptions and the reovirus dsRNA end-labeling reactions were monitored by PAGE to confirm the integrity of the products.

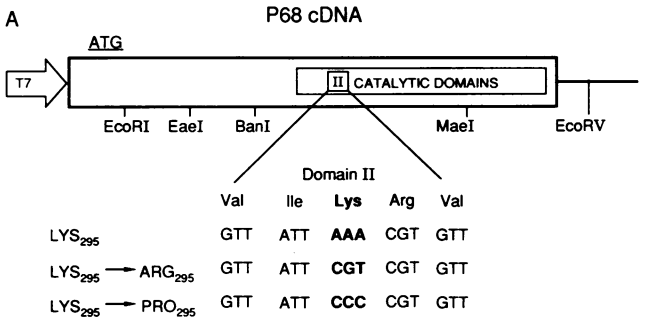
Binding assays. p68 DNA constructs were transcribed and translated as described above. The relative amount of each protein was estimated by laser densitometry scanning of the autoradiogram. Equimolar amounts of the p68 proteins were then reacted with the p68 MAb prior to the binding assay. We estimate that approximately 2 to 5 pmol of p68 protein was reacted with the RNAs in these assays. As a negative control, the P68 MAb was reacted with a translation mix which was not programmed with p68 RNA and subsequently reacted with the radiolabeled RNAs. To ensure that equal moles were reacted with the dsRNAs, we included [^{35}S]methionine in the translation mix. We then electrophoresed an aliquot and measured the relative amounts of the truncated and full-length polypeptides made in vitro. To calculate the moles added, it was necessary to correct for the number of methionines present in the protein. The full-length p68 has nine methionine residues; the *Mae*I, *Ban*I, *Eae*I, and *Eco*RI constructs have eight, five, four, and two, respectively. We also corrected for the different sizes of the constructs (Fig.

1). After correction for the numbers of both methionine residues and amino acids in the different-size proteins, the predicted ratio of the band intensity representing the radiolabeled proteins immunoprecipitated by the p68 Mab and presented to the dsRNAs should be 1.0 (*Eco*RV and Pro-295):0.62 (*Mae*I):0.24 (*Ban*I):0.12 (*Eae*I):0.04 (*Eco*RI). The actual values were 1.0:0.69:0.20:0.10:0.03, thus ensuring that approximately equal moles of the proteins were reacted with the dsRNAs.

After immunoprecipitation and the final buffer II wash, the p68-MAb-Sepharose complex was resuspended in binding buffer (10 mM Tris-HCl [pH 7.5], 100 mM KCl, 0.1 mM EDTA, 2 mM $MgCl_2$, 2 mM $MnCl_2$, 7 mM β-mercaptoethanol, 100 U of aprotinin per ml, 10 μM ATP, 10 μg of tRNA per ml, 0.5% Triton X-100, 20% glycerol). Radiolabeled VAI RNA or reovirus dsRNA was added to each reaction mixture, incubated at 30°C for 20 min, and washed in buffer II containing 0.5% Triton X-100. After the last wash, the pellet was resuspended in NET (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.05% Nonidet P-40), carrier tRNA, and SDS (1%). After phenol-chloroform extraction and ethanol precipitation, RNAs specifically bound to p68 were analyzed on either an 8% polyacrylamide–7 M urea gel (for VAI RNA) or a 10% SDS-polyacrylamide gel (for reovirus dsRNAs [46]). The binding of these RNAs to native p68 (immunopurified from interferon-treated 293 cells) was performed as earlier described (24).

To determine the relative RNA binding of the different p68 constructs, increasing amounts of ^{32}P-labeled reovirus dsRNAs and VAI RNA were incubated with equivalent moles of the desired protein. RNA binding was determined by gel electrophoresis of the reovirus dsRNAs and VAI RNA as described above. The relative amount of binding was determined by scanning the desired RNA species by laser densitometry of the autoradiogram, which gave a numerical value for the area under the curve. For the reovirus dsRNA study, we measured the binding of the S class of RNAs to p68 and determined the average of these values at each RNA concentration tested. The maximal detected binding of the RNA was given the arbitrary value of 100, with other values directly compared against this number.

Oligonucleotide-directed site-specific mutagenesis. Site-specific mutagenesis was performed essentially as described by Kunkel (29), using the Muta-Gene M13 In Vitro kit (Bio-Rad). Single-stranded uracil-containing templates corresponding to the anticoding sequence of the kinase were prepared by using *Escherichia coli* CJ236 (*dut ung*). This template was subjected to mutagenesis using the degenerate oligonucleotide 5′-GAAAGACTTACGTTATTNNNCGTG TTAAATATAATAA-3′, where N is A, C, G, or T (25% of each in the synthesis reaction); thus, the lysine in this position can be mutated to any amino acid or stop codon. The purified oligonucleotides were annealed to uracil-containing DNA templates and used as primers for mutagenesis. Identification of mutant plaques was performed by sequence analysis using the dideoxy-chain termination method (44). Similarly, site-specific mutagenesis was used to create p68 deletion mutants by changing the *Eco*RI and *Eae*I restriction sites (Fig. 1) to *Ban*I sites. Following removal of the *Eco*RI (*Ban*I)-*Ban*I and *Eae*I(*Ban*I)-*Ban*I fragments, p68-specific sequences were then religated such that they would code for p68 proteins (44,000 and 51,000 Da in size, respectively) consisting of part of the amino terminus fused, in frame, to the remainder of the p68 carboxyl region.



B

Restriction Enzyme	# Nucleotides in runoff transcript	# Amino Acids in Protein	Apparent Mr (kd)	# Catalytic Domains
EcoRI	297	90	10	0
EaeI	464	155	17	0
BanI	753	242	30	0
MaeI	1181	384	45	5
EcoRV	1818	550	68	11

FIG. 1. Description of the p68 pcDNAI/NEO vector and wild-type, mutant, and truncated p68 proteins. (A) Schematic representation of the p68 cDNA clone showing the relative locations of the initiation of T7 transcription, the initiator ATG methionine, locations of selected restriction sites used for preparation of the truncated proteins, and the catalytic domains. Also depicted are the conserved amino acid sequences present in domain II (including the wild-type Lys-295) and sequences of the Arg-295 and Pro-295 mutants. (B) Characterization of full-length (*Eco*RV) and truncated p68 RNAs and polypeptides.

RESULTS

In vitro transcription-translation of the 68,000-M_r interferon-induced protein kinase. To express the p68 protein kinase in an in vitro transcription-translation system, the gene encoding the full-length p68 (38) was subcloned into the pcDNAI/NEO expression vector (Fig. 1). Capped p68 RNA of the proper size was prepared by linearizing the DNA with *Eco*RV and transcribing the DNA with T7 polymerase (data not shown). Initial attempts to translate the p68 mRNA in commercially prepared rabbit reticulocyte lysate resulted in unacceptably low yields of p68 protein (data not shown). We therefore prepared our own lysate and assayed the p68 mRNA in the presence of increasing concentrations of the drug 2AP. The 2AP, an inhibitor of the p68 protein kinase (5, 9), was included in the reaction mix to improve yields by preventing possible activation of the endogenous rabbit kinase or the exogenously synthesized human kinase. The presence of 10 mM 2AP in the translation mix enabled us to increase the amounts of the p68 kinase produced in the lysate by almost 20-fold, as determined by laser scanning densitometry of the p68 protein (Fig. 2A). Further, in contrast to translation reactions using commercially prepared lysates (38), the majority of the p68 kinase was expressed as a full-length 68,000-Da product which comigrated with the native p68. Since future experiments required the use of antibody prepared against p68, we tested the reactivity of the in vitro-synthesized kinase with both the MAb (31) and polyclonal antibody (30) prepared against p68. Both types of antibodies efficiently recognized and immunoprecipitated the p68 kinase, with the MAb being more reactive (Fig. 2B; compare lanes A and C). It is relevant to

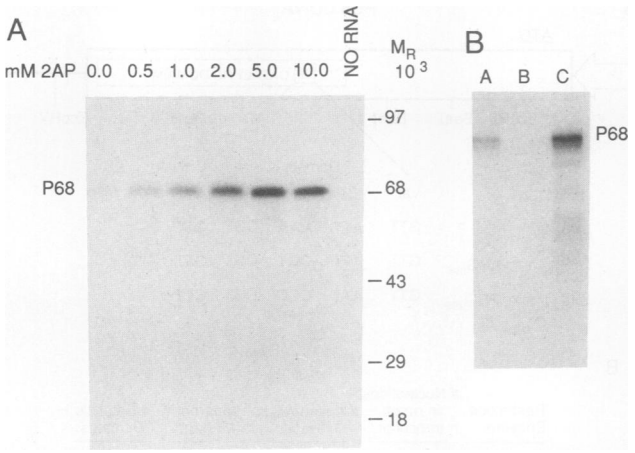


FIG. 2. In vitro synthesis of the full-length P68 protein kinase. (A) Approximately 0.5 μg of in vitro-synthesized p68 RNA was used to program the rabbit reticulocyte extracts in the presence of [^{35}S]methionine. Where indicated, 2AP was included in the translation mix at the indicated concentration. The lane marked NO RNA refers to a translation done in the presence of 10 mM 2AP and in the absence of exogenously added P68 RNA. The translation products were electrophoresed on a 10% SDS-polyacrylamide gel and then subjected to autoradiography. Positions of molecular weight markers are shown at the right. (B) p68 translation products synthesized in the presence of 10 mM 2AP were immunoprecipitated by either the polyclonal (lane A) or monoclonal (lane C) antibody to p68 as described in Materials and Methods. As control, the in vitro-synthesized p68 was reacted with protein A-agarose alone (lane B).

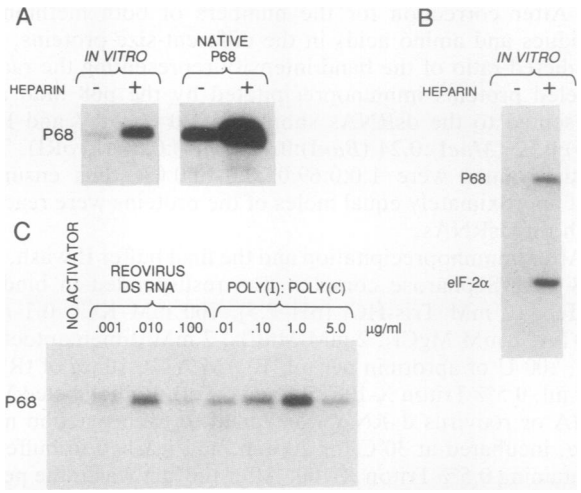


FIG. 3. Autophosphorylation and activity of the in vitro-synthesized p68 protein kinase. (A) In vitro p68 (labeled IN VITRO), synthesized in the presence of 10 mM 2AP, was immunoprecipitated by the p68 MAb and autophosphorylated in the presence of heparin (10 U/ml) where indicated and as described in Materials and Methods. Lanes marked NATIVE P68 refer to an activation reaction performed with p68 immunopurified from interferon-treated 293 cells. Phosphorylated products were analyzed by 10% SDS-PAGE. (B) Activity of the in vitro p68, synthesized in the presence of 10 mM 2AP, immunoprecipitated by the p68 MAb, and incubated in the presence of 0.5 μg of purified eIF-2 and in the presence or absence of the activator heparin at 10 U/ml. (C) Autophosphorylation of the in vitro-synthesized (in the presence of 10 mM 2AP) p68 in the presence of the indicated concentrations of reovirus dsRNA or poly(I-C).

note that the MAb is species specific and recognizes only the human and not the rabbit p68 kinase (23a, 31, 38).

Autophosphorylation and activity of the in vitro-synthesized p68 protein kinase. To perform a structure-function analysis of p68, it was essential to demonstrate that the in vitro-made kinase could be activated (autophosphorylated) in vitro and subsequently phosphorylate its natural substrate, the alpha subunit of eIF-2. We assayed for p68 activation in the presence of 2AP and in the presence or absence of heparin, an effective activator of p68 (16, 17). Following in vitro p68 synthesis, the translation extracts were subjected to immunoprecipitation utilizing the MAb to p68. Any residual 2AP thus would be removed during the immunoprecipitation and subsequent washing steps. The results (Fig. 3A) demonstrated that efficient activation of the kinase was achieved when the translation was performed in the presence of 10 mM 2AP. These results are in agreement with those described above, which showed that maximal synthesis (when there should be the least activation of p68) also occurred at 10 mM 2AP. At lower concentrations of 2AP, extensive phosphorylation of p68 was observed in the absence of added heparin (data not shown). It should be stressed that different preparations of reticulocyte lysate respond differently to the 2AP; each batch should be titrated separately against 2AP to determine the drug concentration for optimal p68 synthesis and activation.

Since the p68 kinase has two activities, we next tested the second activity, the ability to phosphorylate its natural substrate, the alpha subunit of eIF-2. Again similarly to the native kinase, p68 efficiently phosphorylated eIF-2α in response to activation by heparin (Fig. 3B). Although heparin is an effective p68 activator, it was nonetheless essential to show that dsRNAs such as the synthetic poly(I-C) and natural reovirus dsRNAs also could activate the in vitro-synthesized kinase. The kinase was autophosphorylated most efficiently at concentrations of 1 μg of poly(I-C) per ml and 0.01 μg of reovirus dsRNA per ml (Fig. 3C). As previously reported for the native p68 (9, 12, 18), high concentrations of both types of dsRNA inhibited activation of the kinase.

Functional characterization of p68 kinase molecules containing mutations in catalytic domain II. The deduced amino acid sequence of p68 (38) predicted a protein containing all of the domains specific for members of the protein kinase family, including the catalytic domains characteristic of serine/threonine kinases (13). To start to identify active sites present in the p68 protein kinase and at the same time produce a nonfunctional enzyme, we introduced single amino acid substitutions in catalytic domain II at amino acid residue 295. This invariant lysine, which is the best-characterized catalytic domain residue, is directly involved in ATP binding and the phosphotransfer reaction (13). By using site-directed mutagenesis with degenerate oligonucleotides, lysine 295 was changed to either an arginine or proline (Fig. 1). We first analyzed the synthesis of mutant kinase molecules by using the in vitro transcription-translation system and confirmed that both mutants were efficiently made and precipitated by the p68 MAb (Fig. 4A). The incomplete p68 protein products are likely due to partial mRNA degradation, which is sporadically observed in the reticulocyte lysates. To test whether these amino acid substitutions resulted in losses in protein kinase activity, an activation assay was performed on the in vitro-made kinase molecules (Fig. 4B). While the wild-type control was readily activated

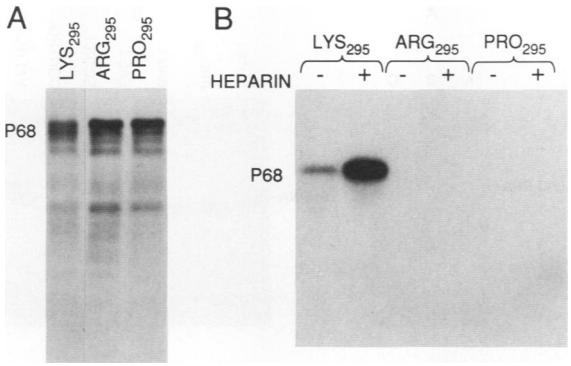


FIG. 4. Synthesis and characterization of p68 kinase molecules with single amino acid substitutions in catalytic domain II. (A) Immunoprecipitation analysis of protein products from a translation mix programmed with 0.25 μg wild-type and mutant RNAs. (B) Autophosphorylation reaction of the in vitro-synthesized mutant and wild-type kinases in the presence or absence of heparin (10 U/ml).

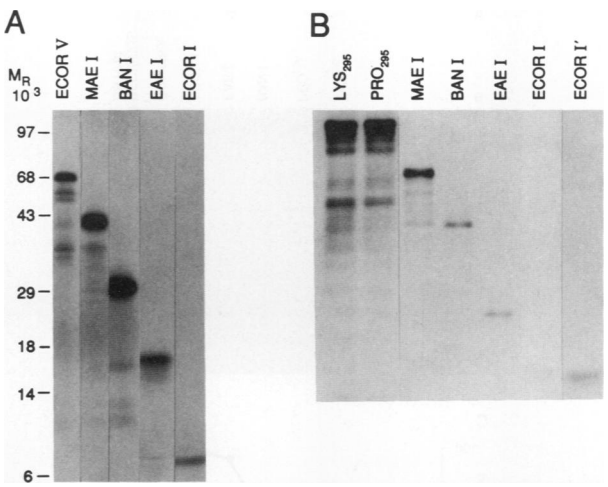


FIG. 5. Synthesis and characterization of truncated p68 kinase molecules. (A) Runoff RNA transcripts (1.0 μg) synthesized in vitro from p68 cDNA restricted with the indicated enzymes (see also Fig. 1) were used to program the rabbit reticulocyte system. Translation products were analyzed by 17% SDS-PAGE and autoradiography. Positions of molecular weight markers are shown at the left. (B) Immunoprecipitation analysis of equimolar amounts of the truncated p68 proteins from a translation mix. Lane *Eco*RI′ is a longer exposure of lane *Eco*RI (6 days versus 2 days) to allow for visualization of the band. See text and Materials and Methods for details of precipitation and quantitation.

by heparin, no autophosphorylation of the mutants was observed even after a 10-times-longer exposure of the autoradiogram. The mutants also failed to phosphorylate eIF-2α, and poly(I-C) and reovirus dsRNAs (as well as heparin) failed to activate the mutant kinases (data not shown).

Functional characterization of truncated p68 kinase molecules. To define regions of p68 which are important not only for function but also for binding to dsRNA activators and viral repressors, we examined kinase proteins which lacked various portions of their carboxyl terminus. The restriction sites used to prepare the shortened RNA transcripts are shown in Fig. 1 along with the predicted sizes of the RNAs and p68 polypeptides and the number of catalytic domains present. Figure 5A shows translation products of the different-size p68 RNAs in reticulocyte lysate. Before analyzing the binding of RNAs to the p68 constructs, we confirmed that the shortened translation products lacking all or some of the critical catalytic domains were not autophosphorylated. Neither the protein product of the *Ban*I construct nor that of the *Mae*I construct, which contain none or five of the catalytic domains, respectively, were phosphorylated in the presence of heparin (data not shown).

We then tested whether the different p68 wild-type and mutant constructs bound to dsRNA activators. We chose to study the binding of reovirus dsRNAs to the kinase since these RNAs are efficient activators of p68, can be end labeled with [^{32}P]ATP, and thus can be readily analyzed on a polyacrylamide gel. The p68 kinase molecules were synthesized in vitro, immunoprecipitated with the MAb, and reacted with ^{32}P-labeled reovirus dsRNAs as described in Materials and Methods. It was important to react equimolar amounts of p68 proteins with the reovirus dsRNAs, and therefore [^{35}S]methionine was included in the translation reactions for accurate quantitation. We then determined the relative amounts of protein synthesized and verified that equal moles were made after correcting for the numbers of both methionines and amino acids present as described in the Materials and Methods. Finally, it was essential that equal moles of the different-size proteins be present in the immunoprecipitates which were subsequently reacted with the dsRNAs. An aliquot of each immunoprecipitate was therefore electrophoresed on a 17% SDS-polyacrylamide gel (Fig. 5B). The intensity of the [^{35}S]methionine signal of the p68 truncated polypeptides decreased according to the predicted values described in Materials and Methods (due to both the reduced methionine content and size of the constructs). After we were satisfied that equal moles of each were bound to the MAb, the binding reaction was performed. Parenthetically, it is of interest that the p68 MAb precipitated each of the truncated polypeptides, even the *Eco*RI construct, which is approximately 10,000 Da, or only about one-seventh the size of the full-length protein (Fig. 5B). These data strongly suggest that the MAb recognizes an epitope close to the p68 amino terminus. Indeed, p68 constructs lacking portions of the amino terminus failed to be immunoprecipitated by the MAb (23a). We cannot rule out, in this binding analysis, that the MAb itself may interfere in some way with RNA binding at the very amino terminus, although the majority of binding appears to occur between the *Eco*RI and *Ban*I restriction sites (see below).

After the binding reaction and appropriate washing, bound reovirus RNAs were electrophoresed on a 10% SDS-polyacrylamide gel. As a control, we tested binding of the dsRNA to the native p68 prepared from interferon-treated 293 cells. RNAs of all size classes efficiently bound to the native p68 as predicted (Fig. 6A). Binding of the reovirus dsRNAs to the in vitro-made kinases (Fig. 6B) was quantified by laser densitometry tracing of the individual bands. Several points can be made regarding this experiment: (i) reovirus dsRNAs bound with equal efficiency to the in vitro-made mutant Pro-295 and wild-type kinase molecules, although only the latter could be activated by these RNAs (similar results were obtained with the Arg-295 mutant; data not shown); (ii) binding varied according to the size of RNAs, with approximately 15 to 20% of the smaller RNAs and 2 to 5% of the larger RNAs binding to the mutant and wild-type p68; (iii) reovirus dsRNAs bound to the *Ban*I-derived protein in somewhat reduced amounts, as described

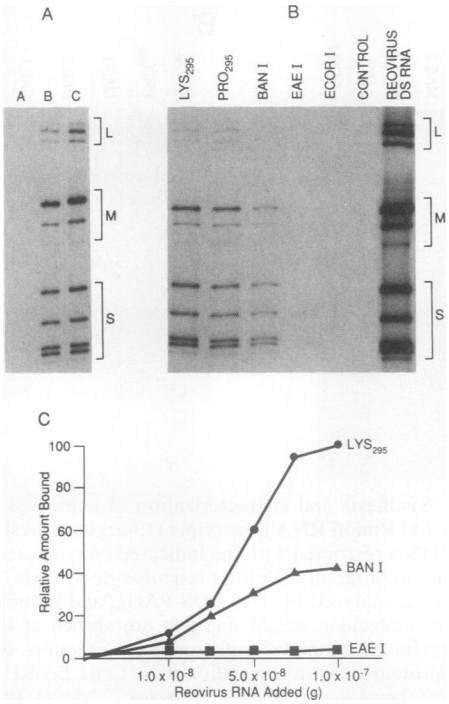


FIG. 6. Binding of reovirus dsRNAs to wild-type, mutant, and truncated p68 kinase molecules. (A) As control to test the binding to native p68, ^{32}P-labeled reovirus dsRNA (50 ng) was added to MAb-Sepharose alone (lane A) or to MAb-Sepharose that was prereacted with an interferon-treated 293 cell extract to purify P68 (lane B). Lane C contains 25 ng of starting material of reovirus dsRNA. Reovirus dsRNAs were analyzed by 10% SDS-PAGE. Migration of large (L; ca. 3,500 to 3,900 nucleotides), medium (M; ca. 2,200 to 2,300 nucleotides), and small (S; ca. 1,200 to 1,400 nucleotides) reovirus dsRNA species are shown at the right. (B) ^{32}P-labeled reovirus dsRNAs (50 ng) were added to equimolar amounts of the wild-type, mutant, and truncated p68 proteins indicated above the lanes, and bound RNAs were analyzed as described in Materials and Methods. Lane CONTROL refers to the negative control showing binding of RNAs to the MAb-Sepharose which was prereacted with a translation mix not programmed with p68 RNA. Lane REOVIRUS dsRNA contains 25 ng of the starting material. (C) Analysis of the RNA binding to the *Eco*RV-, *Ban*I-, and *Eae*I-derived constructs. Increasing concentrations of radiolabeled reovirus dsRNAs were reacted with equivalent moles of these proteins. The relative amount bound was determined as described in Materials and Methods.

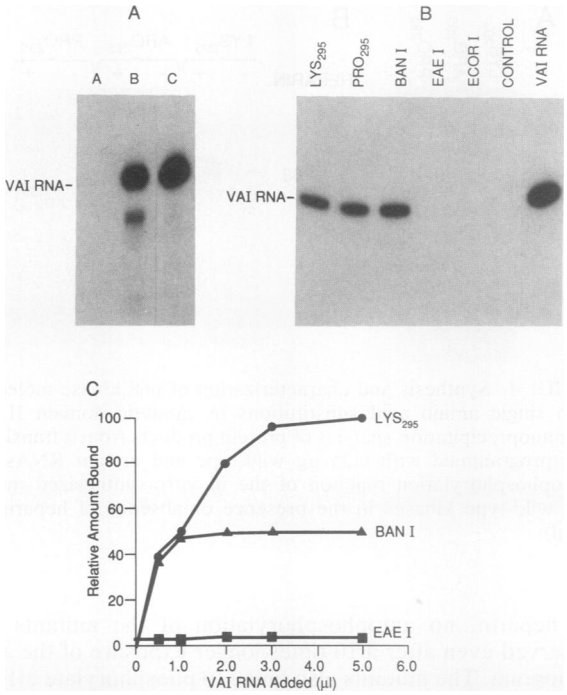


FIG. 7. Binding of VAI RNA to p68 proteins. (A) As a control to test the binding to native p68, in vitro-transcribed ^{32}P-labeled adenovirus VAI RNA (1.0 µl; 0.050 µg/µl) was added to MAb-Sepharose alone (lane A) or to MAb-Sepharose prereacted with an interferon-treated 293 cell extract to purify p68 (lane B). Lane C contains 0.25 µl of the starting material. VAI RNA was analyzed on an 8% acrylamide–7 M urea denaturing gel. (B) Radiolabeled VAI RNA (1.0 µl) was added to equimolar amounts of the wild-type, mutant, and truncated p68 proteins indicated above the lanes, and bound RNA was analyzed as described in Materials and Methods. Lane CONTROL refers to negative control showing binding of VAI RNA to MAb-Sepharose which was prereacted with a translation mix not programmed with p68 RNA. Lane VAI RNA depicts 0.25 µl of the starting material. (C) Analysis of VAI RNA binding to the *Eco*RV-, *Ban*I-, and *Eae*I-derived constructs. Increasing amounts of radiolabeled VAI RNA were reacted with equivalent moles of these constructs, and binding was analyzed as described in Materials and Methods.

below in more detail; and (iv) no detectable RNA bound to either the *Eae*I or *Eco*RI p68 construct.

To confirm these results and determine the relative amounts of RNA binding of selected constructs, the following experiments were performed. We reacted equal moles of the full-length p68 and the *Ban*I- and *Eae*I-derived constructs with increasing concentrations of radiolabeled reovirus dsRNAs. Binding reactions were performed, and the relative amount bound of representative reovirus dsRNAs was determined as described in Materials and Methods. The results show that while there was little binding to the *Eae*I p68, there was extensive binding to the full-length *Eco*RV construct, leveling off at the higher RNA concentrations. We also found nearly identical binding of the mutant and *Mae*I p68-derived proteins compared with the full-length p68 (data not shown). In contrast, while binding to the *Ban*I-derived protein was similar to binding to the full-length p68 at low RNA concentrations, the binding plateaued earlier.

We next performed similar binding studies with the adenovirus-encoded VAI RNA, which has been shown to complex with and inactivate the p68 kinase both in vivo and in vitro (12, 24, 36). These experiments were initiated to determine the molecular mechanisms of inhibition and whether VAI RNA and dsRNA activators bound to the same regions of the p68 kinase. ^{32}P-labeled bona fide VAI RNA was transcribed from a plasmid containing a T7 promoter immediately upstream of the VAI RNA start site and an *Eco*RI site next to the terminator sequence. The in vitro transcription product differed from that of virus-infected cells only in the addition of an extra U at the 3′ end (10, 47). As control, we first confirmed the specific binding of VAI RNA to the native p68 kinase (Fig. 7A). VAI RNA also bound to the in vitro-made wild-type, mutant, and *Ban*I-derived proteins (approximately 2% of added VAI RNA bound; Fig. 7B). Notably, VAI RNA did not bind above background levels to the shorter p68 kinase molecules, similar to the reovirus dsRNAs. We also examined VAI

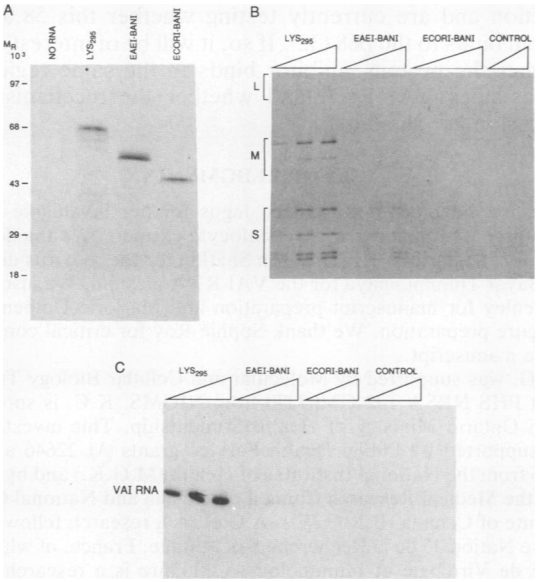


FIG. 8. Binding of reovirus dsRNAs and VAI RNA to amino-terminal deletions of p68. (A) In-frame *Eco*RI-*Ban*I and *Eae*I-*Ban*I deletions were constructed as described in Materials and Methods. RNA synthesized from these deletions and from the full-length wild-type p68 (LYS_{295}) were used to program the rabbit reticulocyte extract. The in vitro-made proteins were then immunoprecipitated with the p68 MAb and analyzed by 14% SDS-PAGE and autoradiography. Lane NO RNA shows in vitro translation and immunoprecipitation of extracts to which no RNA was added. Positions of molecular weight markers are shown at the left. (B) Equimolar amounts of the full-length (LYS_{295}) and deleted p68 proteins were reacted with increasing amounts of radiolabeled reovirus dsRNAs (0.025, 0.050, and 0.100 μg) as described for the truncated constructs. Lanes CONTROL refer to the negative control showing binding of the RNAs (0.050 and 0.100 μg) to MAb-Sepharose which was prereacted with a translation mix not programmed with p68 RNA. (C) Increasing amounts of in vitro-transcribed ^{32}P-labeled VAI RNA (1.0, 2.0, and 4.0 μl) were reacted with the deletion constructs and full-length p68. Lane CONTROL refers to binding of the VAI RNA to MAb-Sepharose which was prereacted with a translation mix not programmed with p68 RNA.

RNA binding in an analysis identical to that performed above for reovirus dsRNA (Fig. 7C). Again we found that at low RNA concentrations, VAI RNA bound equally to the full-length and *Ban*I-derived proteins (explaining the equal signal in Fig. 7B). However, similar to the reovirus RNA binding, at higher RNA levels the binding to the shorter construct plateaued earlier. There was no detectable VAI RNA binding to the *Eae*I-derived protein at any RNA concentration.

To provide additional evidence that the p68 amino terminus contained the regions critical for RNA binding, the following deletion analysis was performed. By using site-specific mutagenesis as described in Materials and Methods, *Eco*RI-*Ban*I and *Eae*I-*Ban*I fragments were removed from the p68 gene (Fig. 1). The deleted constructs gave rise to p68-specific in-frame fusion proteins after in vitro translation of approximately 44,000 and 51,000 Da, respectively, which were precipitable by the p68 MAb (Fig. 8A). Equal moles of the deleted constructs were then reacted with increasing amounts of radiolabeled reovirus dsRNAs or VAI RNA as earlier described. The *Eae*I-*Ban*I deletion bound approximately 5- to 10-fold less reovirus dsRNA than did the full-length wild-type construct, while the *Eco*RI-*Ban*I construct bound barely detectable levels of reovirus dsRNA (Fig. 8B). Neither deletion mutant bound appreciable levels of VAI RNA compared with the full-length p68 (Fig. 8C). These results confirm our previous truncation analysis and demonstrate that both reovirus dsRNA and VAI RNA binding sites reside in the same region of the p68 amino terminus, primarily between the *Eae*I and *Ban*I restriction enzyme sites.

DISCUSSION

We have optimized an in vitro transcription-translation system in which a functional p68 protein kinase is synthesized. This expression system allows us to perform a detailed structure-function analysis on the interferon-induced kinase, with the first of such studies reported here. For maximal synthesis of p68 in vitro, it was necessary to include the drug 2AP in the translation reaction (Fig. 2). It remains unclear exactly why 2AP, a known inhibitor of the dsRNA-activated protein kinase (5, 9), stimulates p68 synthesis, although autophosphorylation experiments (Fig. 3A) suggest that repression of the activation of the endogenous or exogenously made p68 may be responsible. Activation of the kinase in the reticulocyte lysate can be caused by residual fragments of micrococcal nuclease-treated RNAs or alternatively from the exogenously added p68 RNA itself. Indeed, others have shown that natural single-stranded RNAs can activate the protein kinase through double-stranded regions in their secondary structure (3, 8, 45). It is important to note, however, that the mechanisms of 2AP action are still poorly defined. Recent reports suggested that 2AP may regulate the phosphorylation of initiation factors other than eIF-2 (19) or even block the induction of interferon (35).

Introduction of a single amino acid substitution in domain II at lysine 295 completely eliminated protein kinase activity without disturbing the synthesis of the mutant proteins (Fig. 4). This site in catalytic domain II, preserved among both serine/threonine and tyrosine kinases, appears to be directly involved in phosphate transfer, possibly proton transfer (13, 22). Proof that this residue contains an ATP binding site also was provided by early experiments utilizing the ATP analog *p*-fluorosulfonyl 5′-benzoyl adenosine, which reacted with the invariant lysine present in the $p60^{src}$ and cyclic AMP-dependent protein kinases among others and inactivated activity of these enzymes (23, 53). Further, mutagenesis analysis of this lysine in other systems revealed that, similar to our results, substitution by even an arginine residue abolishes protein kinase activity, although in some systems a very low amount of activity can be detected (14, 15, 48, 51). The availability of an inactive p68 protein kinase permits us to dissect further the different functions of the protein kinase, including whether excessive autophosphorylation of the in vitro-made kinase compromises its own synthesis in reticulocyte extract. Preliminary experiments, using transient transfection assays with the mutant and wild-type p68 cDNAs, suggest that this may be the case since mutant p68 proteins are synthesized at least 10-fold more efficiently than is wild-type p68 (2a).

To properly study the molecular mechanisms of kinase activation and repression, it was essential to identify regions of the p68 kinase which interact with RNA activators and viral inhibitors such as VAI RNA. For some years it has been a mystery why the p68 kinase is activated by low and inhibited by high concentrations of dsRNA (reviewed in reference 16). Recent data suggest that this difference results

from the presence of high- and low-affinity binding sites for dsRNA (12) but this view remains to be definitively proven. Moreover, it is still controversial whether kinase autophosphorylation results from intermolecular (28) or intramolecular (12) events. p68 does not possess the ribonucleoprotein consensus sequences (6) typical of RNA-binding proteins, although the sequence indicates the kinase is a hydrophilic protein, which may account for its ability to bind RNA (38). To begin to define RNA binding regions, we prepared truncated kinase molecules lacking various portions of the enzyme's carboxyl terminus and catalytic domains and deleted molecules lacking portions of the amino terminus. Our data suggest that dsRNA binds primarily to the amino, noncatalytic half of the p68 molecule (Fig. 6), particularly since relatively little RNA bound to the *Eae*I-*Ban*I and *Eco*RI-*Ban*I deletion constructs (Fig. 8). In support of these observations, with use of poly(I-C) cellulose and truncated p68 mutants containing either the amino (noncatalytic) or carboxyl (catalytic) regions, it was found that only the constructs containing the noncatalytic portions of p68 efficiently bound to the poly(I-C)-cellulose (4a). We are currently performing additional experiments to more precisely identify the RNA binding domains. We would like to point out that these results do not absolutely rule out the presence of dsRNA binding sites in the carboxyl terminus, as multiple sites may exist throughout the enzyme. Indeed, less reovirus dsRNAs and VAI RNA bound (at high RNA concentrations) to the *Ban*I-derived protein, suggesting that binding sites 3′ to the *Ban*I site may exist. However, it may be just as likely the reduced reovirus dsRNA and VAI RNA binding to the *Ban*I truncated molecules resulted from the improper folding and structure of the *Ban*I-derived protein, which represents less than half of the intact p68 kinase.

As earlier stated, animal viruses have devised methods to downregulate the protein kinase and avoid the negative effects on protein synthesis initiation. The best-characterized viral inhibitor is the adenovirus-encoded RNA polymerase III product, VAI RNA, which has been shown to repress p68 activity by complexing to the kinase (12, 24, 36). VAI RNA, however, binds with reduced efficiency compared with dsRNA activators (12, 28) and, in order to mediate the repression of p68 in vitro, must be present before the addition of dsRNA (12). Similarly, we have found that less VAI RNA than reovirus dsRNAs binds to the in vitro-made kinase. The reduced binding is likely due to the imperfect dsRNA structure of the VAI RNA (10). The binding analysis nevertheless suggests that VAI RNA functions by interacting with regions of p68 similar to those with which reovirus dsRNAs interact (Fig. 6 to 8). Whether VAI RNA-induced repression results from a direct competition for available RNA binding sites or from a change in p68 conformation remains to be determined. It is relevant to note that the p68 kinase isolated from adenovirus-infected cells is irreversibly inactivated, suggesting that the kinase may have been altered during infection in some way (24). That both VAI RNA and dsRNA bind to the amino terminus of the kinase, predominantly between the *Eae*I and *Ban*I restriction sites, suggests that this region represents a regulatory region of considerable importance that is distinct from the catalytic domains located at the carboxyl end. Both protein kinase C and cyclic GMP-dependent protein kinases also contain regulatory domains located at their amino termini which are distinct from their catalytic domains (reviewed in reference 49). Finally, it is important to note that not all inhibitors of p68 are RNAs. We recently have identified a cellular protein inhibitor of p68 that is activated during influenza virus infection and are currently testing whether this 58,000-Da protein binds to the p68 (32). If so, it will be of interest to test whether the protein inhibitor binds to the same regions of p68 as does VAI RNA and whether the mechanisms of repression are similar.

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