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Autophosphorylation Sites Participate in the Activation of the Double-Stranded-RNA-Activated Protein Kinase PKR

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The interferon-induced RNA-dependent protein kinase PKR is found in cells in a latent state. In response to the binding of double-stranded RNA, the enzyme becomes activated and autophosphorylated on several serine and threonine residues. Consequently, it has been postulated that autophosphorylation is a prerequisite for activation of the kinase. We report the identification of PKR sites that are autophosphorylated in vitro concomitantly with activation and examine their roles in the activation of PKR. Mutation of one site, threonine 258, results in a kinase that is less efficient in autophosphorylation and in phosphorylating its substrate, the initiation factor eIF2, in vitro. The mutant kinase is also impaired in vivo, displaying reduced ability to inhibit protein synthesis in yeast and mammalian cells and to induce a slow-growth phenotype in *Saccharomyces cerevisiae*. Mutations at two neighboring sites, serine 242 and threonine 255, exacerbated the effect. Taken together with earlier results (S. B. Lee, S. R. Green, M. B. Mathews, and M. Esteban, Proc. Natl. Acad. Sci. USA 91:10551–10555, 1994), these data suggest that the central part of the PKR molecule, lying between its RNA-binding and catalytic domains, regulates kinase activity via autophosphorylation.

The RNA-dependent protein kinase PKR, also known as DAI, is an interferon-induced protein that plays important roles in several regulatory processes (for a review, see reference 10). First recognized as an inhibitor of translation (14) and a mediator of the host antiviral response (reviewed in reference 40), PKR is also implicated in cellular growth control (9), tumor suppression (29, 45), cellular differentiation (51), apoptosis (35), and signal transduction (48, 69). The enzyme is found in most mammalian cells at a basal level and in an inactive or latent state. Upon activation by double-stranded RNA (dsRNA), it phosphorylates the translational initiation factor eIF2 on serine 51 of its α subunit (14, 53, 54). Protein synthesis is then halted at the stage of initiation because of the entrapment of the guanosine nucleotide exchange factor eIF2B (for a review, see reference 43). Several viruses have evolved mechanisms that overcome the PKR-mediated antiviral response (40). PKR can also phosphorylate other substrates, including IκB (31, 49), histone H2A (17), an unidentified 90-kDa protein (55), and human immunodeficiency virus Tat (5, 42), but the consequences of these phosphorylation events are less well established.

PKR belongs to a small family of eIF2 kinases which modify the same site but are regulated differently. In mammals, the heme-regulated inhibitor HRI is found in reticulocytes, where protein synthesis is dependent on the availability of heme. HRI is inactivated by the binding of heme; thus, when heme is absent, the kinase is active and globin synthesis is inhibited (8). GCN2 is a third eIF2 kinase, found in the yeast *Saccharomyces cerevisiae* (12, 24). It is regulated by amino acid deprivation, and its activator is uncharged tRNA (for a review, see reference 25). Limited phosphorylation of eIF2 by GCN2 induces the synthesis of the transcriptional activator GCN4 through the suppression of initiation at an upstream open reading frame in the GCN4 mRNA. GCN4 stimulates transcription from multiple amino acid biosynthetic genes, thereby increasing the biosynthetic capacity of the cell and reversing amino acid limitation. Whereas a low level of eIF2 phosphorylation derepresses GCN4 synthesis, a high level of eIF2 phosphorylation strongly inhibits overall protein synthesis and negatively affects cell growth in *S. cerevisiae* (9, 11), as in other cells.

The catalytic domains of the three eIF2 kinases share extensive homology (8), but the regulatory domains differ. The catalytic domain of PKR, containing the 11 conserved kinase subdomains, extends from amino acid 273 to the protein's C terminus at amino acid 551 (22, 44, 64). Located within this domain is the ATP-binding region including the requisite lysine residue at position 296 (44). PKR possesses an RNA-binding domain, which spans the first 171 amino acids of its N terminus and is composed of two distinct copies of an RNA-binding motif (dsRBM) which are rich in basic amino acids and are separated by a short spacer (7, 15, 20, 21, 27, 50, 63). Lying between the RNA-binding and catalytic domains is the central portion of the protein, which is very rich in serine and threonine residues and contains a third basic region (amino acids 233 to 268). This central portion of the molecule does not seem to participate in RNA binding (21), and its function is unknown. Nevertheless, deletion of residues 234 to 272 resulted in a kinase that was inactive when tested in higher cells with a vaccinia virus-based assay (36) and a yeast growth assay (58).

PKR becomes autophosphorylated when bound to its activator, dsRNA, and it has been proposed that autophosphory-

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lation is required for activation. Supporting this view, the level of phosphate incorporation into PKR in vitro has been correlated with its substrate-phosphorylating activity (3, 18). Moreover, many inhibitors of PKR activation, including adenovirus VA RNA_I, vaccinia virus K3L, and the cellular protein P58, also prevent its autophosphorylation (reviewed in references 26, 41, and 62). Similarly, both activation and autophosphorylation are suppressed at superoptimal dsRNA concentrations, as well as by short RNA duplexes (38). Despite earlier reports to the contrary (3, 19), recent data indicate that autophosphorylation is an intermolecular reaction (30, 58, 65, 66), consistent with a bimolecular model for PKR activation that accounts for several aspects of the enzyme's dependence on dsRNA (30).

It appears that several serine and threonine residues become phosphorylated during PKR activation (18, 33), but neither the sites of autophosphorylation nor their role in the enzyme's activation have been established. To address these issues, we identified residues that become phosphorylated when PKR is activated in vitro in the presence of dsRNA and then constructed mutant kinases in which the phosphorylation sites were changed to alanine. The effects of the mutations were examined to assess the importance of the sites and the relationship between autophosphorylation and activation. Two clusters of phosphorylation sites were found. One cluster is located in the spacer of the RNA-binding domain; these sites do not appear to affect the PKR activation and will be discussed elsewhere (63a). The second cluster lies in the third basic region; mutation of threonine 258 to alanine reduced (but did not abrogate) PKR function, and the mutation of two additional autophosphorylation sites, serine 242 and threonine 255, further attenuated its kinase activity. Thus, the third basic region may define a second regulatory domain, that of an autoregulatory region which changes the enzyme specificity from an autophosphorylation mode to a substrate phosphorylation mode.

MATERIALS AND METHODS

Labeling of PKR. PKR, purified to the Mono S stage from alpha interferon-treated 293 cells (30); was activated by incubation with reovirus dsRNA (provided by A. Shatkin) in the presence of [γ-^{32}P]ATP, under conditions optimized for maximum labeling of the protein. Reactions were carried out in a 20-μl volume containing 1 μl (10 ng) of kinase in 10 μl of diluent (20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES]-K^+ [pH 7.4], 0.1 mM EDTA, 1 mM dithiothreitol) and 10 μl of 2× kinase mix (0.03 M HEPES-K^+ [pH 7.4]; 6 mM $MgCl_2$; 0.02 μg each of leupeptin, pepstatin, and aprotinin; 2 mM dithiothreitol; 0.02 mM phenylmethylsulfonyl fluoride; 0.08 μg of dsRNA per ml; 20 μCi of [γ-^{32}P]ATP). Reactions were incubated for 30 min at 30°C with 10-μCi additions of [γ-^{32}P]ATP at 7, 10, and 20 min. Twenty reaction mixtures were pooled, and PKR was immunoprecipitated with a rabbit polyclonal antibody (21) by incubation in NET buffer (0.5% Nonidet P-40, 5 mM EDTA, 150 mM NaCl, 50 mM Tris-HCl [pH 7.4], 0.02% sodium azide, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate [SDS]) for 30 min on ice. Protein A-Sepharose (Pharmacia) was added, and incubation was continued for an additional 30 min. Immunoprecipitates were washed five times with 1 ml of NET buffer and once with 1 ml of water.

Generation of radiolabeled peptides. ^{32}P-labeled PKR was eluted from the protein A-Sepharose beads by incubation for 20 min at room temperature in 99% formic acid. The beads were washed twice with formic acid, and the combined washes were lyophilized. The dried eluate was resuspended in 70% formic acid and incubated with 40 μg of cyanogen bromide (Sigma) for 20 h at room temperature in the dark. The solution was dried in a vacuum centrifuge (Savant) with heat, 1 ml of deionized water was added, and the digest was dried again. Finally, 0.5 ml of 100 mM ammonium bicarbonate (prepared just prior to use) was added and the volume was reduced to approximately 50 μl. Soluble phosphopeptides were resolved in step gradient Tris-Tricine-SDS gels (61), fixed, dried, and visualized by autoradiography.

Separation of PKR phosphopeptides. The CNBr digest was subjected to high-performance liquid chromatography (HPLC) through an Aquapore RP 300 C8 column (7 μm, 2.1 by 220 mm; Perkin Elmer/Brownlee) with a Hewlett-Packard 1090M chromatograph. The sample was applied to the column at a flow rate of 0.5 ml/min, and nonadsorbed material was washed through for 30 min. The column was developed with a 0 to 70% (vol/vol) gradient of acetonitrile in 0.085% (wt/vol) trifluoroacetic acid over 50 min. Absorbance of the effluent was monitored at 220 and 280 nm. Fractions (0.5 ml) were collected and monitored by measuring Cerenkov radiation in an LKB scintillation counter.

Radioactive sequence analysis. Radioactive peaks from the HPLC column were pooled. Peptides were lyophilized, resuspended in 60% acetonitrile, and coupled to arylamine-derivatized polyvinylidene difluoride membranes (Sequalon AA; Millipore) with carbodiimide. Polyvinylidene difluoride-bound peptides were subjected to repetitive Edman degradations on a protein sequencer (Applied Biosystems 473A). Amino acid derivatives were collected in scintillation vials as described by Russo et al. (59), and ^{32}P was determined as described above. Peptides from primary digests were analyzed three to six times, and secondary peptides were analyzed twice. Radioactive peaks that exceeded the background by 100 cpm were considered significant unless they were present in the trail from a larger peak.

Lys-C digestion. Secondary digestion of radiolabeled PKR peptides was performed with the endoproteinase Lys-C (sequencing grade; Boehringer Mannheim). Lys-C was dissolved in 0.001 N HCl and stored at −20°C. The lyophilized peptide was resuspended in 40 μl of 0.1% trifluoroacetic acid–30% acetonitrile, and 40 μl of 0.1 M ammonium bicarbonate was added with 2.5 μl of enzyme (1 μg/μl). After incubation for 1 h at 37°C, an additional 2.5 μl of protease was added and the incubation was continued for another 20 h.

Phosphoamino acid analysis. Radiolabeled PKR or peptides were lyophilized, resuspended in 6 N HCl, and hydrolyzed at 110°C for 1 h under vacuum. Insoluble material was removed by centrifugation, and the supernatant was lyophilized. Deionized water (50 μl) was added and lyophilized to dryness. Radiolabeled phosphoamino acids were separated on glass-backed cellulose thin-layer chromatography plates (EM 5716-7) at pH 3.5 (4). Standards (phosphoserine, phosphothreonine, and phosphotyrosine [Sigma]) were mixed with samples and visualized by ninhydrin staining. Radiolabeled phosphoamino acids were visualized by autoradiography.

Mutagenesis. Sites of PKR autophosphorylation were mutated by the oligonucleotide-directed site-specific procedure (1, 70) to generate the following changes: T255A, T258A, S242A, T255/258AA, and S242A/T255/258AA (Triple).

PKR expression in cultured cells. PKR activity was monitored in BSC-40 monkey cells as described previously (36, 37). Fragments harboring the mutations were subcloned into pTL1, a plasmid containing the intact PKR cDNA under the control of the vaccinia virus late promoter p4b and the *E. coli lacI* operator-repressor elements. BSC-40 cells grown in 24-well Limbro plates were infected with vaccinia virus at 2 PFU per cell. One hour later, the cells were cotransfected with 1 μg of pTL1, or its mutant derivatives, and 1 μg of pPR15 (57), which contains the luciferase reporter gene under the control of the p4b promoter. After 3 h, the medium was changed and PKR was induced by addition of 1.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) to two of each set of four cultures. Cell extracts were prepared at 24 h postinfection for determination of luciferase activity (6, 56).

PKR expression in *S. cerevisiae*. PKR mutations were inserted between the *Hin*dIII and *Bam*HI sites of a modified pEMBLyex4 vector carrying the PKR cDNA (58). The plasmids were introduced into yeast strains H1816 and H1817 (12), which have the *gcn2* deletion and wild-type or mutant eIF2, respectively. Transformed cells were grown in glucose-containing synthetic minimal medium (SD) at 30°C for approximately 24 h. A 1:50 dilution of this culture was shifted to inducing conditions with SGR synthetic minimal medium containing 10% galactose and 2% raffinose (instead of glucose) and incubated for 15 h at 30°C. Protein extracts were made by disrupting the cells in 300 μl of lysis buffer (0.1 M Tris [pH 8.0], 20% glycerol, 1 mM β-mercaptoethanol, 0.2 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 1 μg each of leupeptin, aprotinin, and pepstatin per ml) with glass beads. Protein concentrations were determined with a Coomassie blue dye-binding assay (Bio-Rad).

Western blot (immunoblot) analysis. Samples of total protein (15 μg) were resolved in 10% polyacrylamide–SDS gels. Proteins were transferred to nitrocellulose, which was then blocked in 5% nonfat dry milk in TBS-T (20 mM Tris [pH 8.0], 150 mM NaCl, 0.1% Tween 20). Blots were probed with a PKR-specific monoclonal antibody (34) and visualized by chemiluminescence autoradiography (ECL; Amersham).

Kinase assay. Yeast extracts (50 μg of protein) were immunoprecipitated as described above, except that the procedure was conducted in buffer I (20 mM Tris [pH 7.5], 50 mM KCl, 400 mM NaCl, 1% Triton-X-100, 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 20% glycerol, 0.17 μg each of leupeptin, aprotinin, and pepstatin per ml. The immunoprecipitates were washed three times in 1 ml of buffer I, three times in buffer II (15 mM HEPES-K^+, 10 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 0.1 M KCl, 0.17 μg each of leupeptin, aprotinin, and pepstatin per ml), and once in buffer III (buffer II with 3 mM $MgCl_2$ substituted for KCl). The beads were incubated for 20 min at 30°C with 30 μl of kinase buffer (buffer III supplemented with 40 ng of reovirus dsRNA per ml and 500 μCi of [γ-^{32}P]ATP per ml). A 3-μl volume of eIF2 (39) was then added to the beads, and the reaction was allowed to proceed for an additional 20 min. An equal volume of 2× sample buffer (32) was added, and the mixture was heated to 100°C for 5 min. The beads were centrifuged to the bottom of the tube, and the supernatant was removed and analyzed in a 20% polyacrylamide–SDS gel. The gel was fixed, dried, and exposed to Kodak XAR-5 film for autoradiography. Phosphorylation was quantified with a Fuji BioImage analyzer.

RESULTS

PKR autophosphorylation accompanies activation of the kinase. To determine the significance of autophosphorylation in the activation of PKR, residues autophosphorylated during activation in vitro were identified and then mutated; the function of the resultant PKR mutants was assessed in mammalian cells, in *S. cerevisiae*, and in vitro.

Characteristics of PKR-derived peptides. PKR is unusually rich in serine and threonine residues, which account for 87 of the 551 amino acids. It is also rich in basic residues, which precludes digestion with trypsin as the first step in phosphopeptide analysis. Consequently, we used cyanogen bromide to cleave the protein at methionine. This reagent is predicted to give rise to 10 peptides, F1 to F10, ranging in size from 1 to 101 amino acids: the predicted digestion products are diagrammed in Fig. 1A, and their molecular masses are listed in Fig. 1B.

To examine the peptides that are phosphorylated upon activation of PKR, the protein was labeled with [γ-^{32}P]ATP by incubation in the presence of dsRNA, isolated by immunoprecipitation, and digested with cyanogen bromide. Four major radiolabeled peptide bands were resolved in a Tris-Tricine gel (Fig. 1C). The apparent molecular masses of these peptides were 21, 15.5, 10.3, and 3.5 kDa, as determined by an average of two separate experiments. For a number of reasons, the phosphorylated peptides cannot be identified by electrophoretic mobility alone. First, several peptides are predicted to cluster at similar molecular weights; second, phosphorylation of PKR induces retardation in gel mobility (44, 60); third, partial cleavage can occur (see below); and, finally, none of the peptides corresponds exactly to any of the predicted molecular weights. We therefore separated the labeled peptides by reverse-phase HPLC for further analysis. The column profile (Fig. 1D) gave a small peak at about fraction 30 (fraction A), another near fraction 40 (fraction B), and two large peaks at approximately fractions 65 and 86 (fractions C and D).

Size and phosphoamino acid analysis of peptides. The radioactive peaks obtained by HPLC were pooled, and their contents were examined by gel electrophoresis (Fig. 2) and for phosphoamino acid content (Fig. 3). Some of the HPLC peaks were divided into leading and trailing portions (fractions A1, A2, etc.; Fig. 1D), which were pooled and analyzed separately. Fractions A1 and A2 contained a band that resolved at 5.2 kDa (Fig. 2, lanes 3 and 4). Fractions B1 and B2 contained a major radiolabeled peptide band that resolved at an apparent molecular mass of 3.5 kDa; fraction B2 also contained a minor band at 5.2 kDa (lanes 5 and 6). Fraction C contained peptides of 10.3, 15.5, and 21 kDa (lane 7). Further digestion with cyanogen bromide proved that the two more slowly moving bands were the result of partial digestion and contained only the 10.3-kDa peptide as the radiolabeled portion (data not shown). Fractions D1 and D2 also contained a 10.3-kDa peptide together with partial digestion products in different ratios (lanes 8 and 9).

The phosphoamino acid content of the HPLC fractions was analyzed by one-dimensional thin-layer electrophoresis after acid hydrolysis. As expected, full-length PKR yielded phosphoserine and phosphothreonine but no phosphotyrosine (Fig. 3, PKR). All of the fractions except B2 contained both phosphoserine and phosphothreonine; only phosphoserine was detected in fraction B2 (Fig. 3). Taking this information together with the gel results, we can tentatively conclude that the 3.5-kDa peptide contains phosphoserine but no phosphothreonine, while the 5.2- and 10.3-kDa peptides may be phosphorylated on serine and threonine residues.

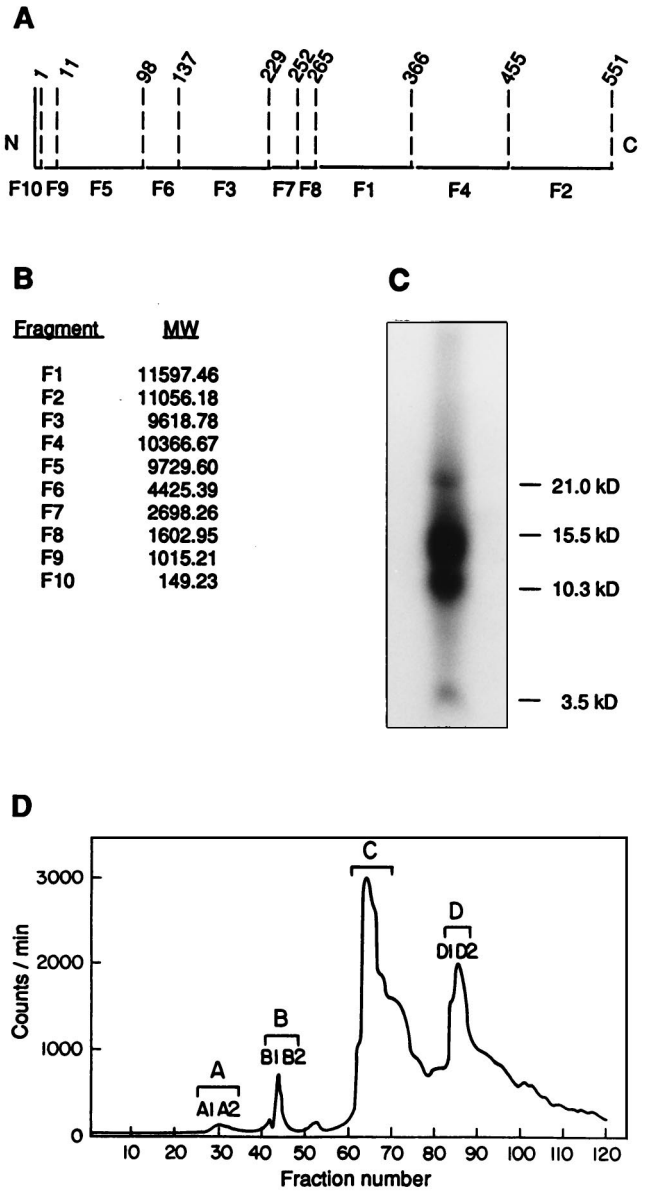

FIG. 1. Cyanogen bromide digestion of autophosphorylated PKR. (A) Sites of cleavage along the PKR molecule (amino acids 1 to 551). Cyanogen bromide cleaves peptide bonds at the C-terminal side of methionine residues. The amino acid numbers of these methionine residues are shown above the linear representation of PKR. Fragments F1 to F10 are numbered in order of decreasing length. (B) Calculated molecular weights (MW) of predicted fragments. (C) PKR, radiolabeled in vitro by autophosphorylation, was digested with cyanogen bromide, and the products were examined by electrophoresis in a Tris-Tricine step gradient gel (10 and 16.5% polyacrylamide–SDS). Labeled peptides were detected by autoradiography. kD, kilodaltons. (D) The digestion products were also examined by reverse-phase HPLC. Fractions (0.5 ml) were collected and assayed for radioactivity by Cerenkov counting.

Phosphopeptide sequence determination. The peptides obtained by HPLC were subjected to sequential Edman degradation in an automated sequencer, and the radioactivity released at each cycle was monitored. The results for fractions A and B are displayed in Fig. 4. Fraction A gave peaks at cycles 3 and 6 (Fig. 4A), which were reproducible despite the low level of radioactivity in this fraction. The low yield at cycle 6 is probably due to the poor efficiency of Edman degradation

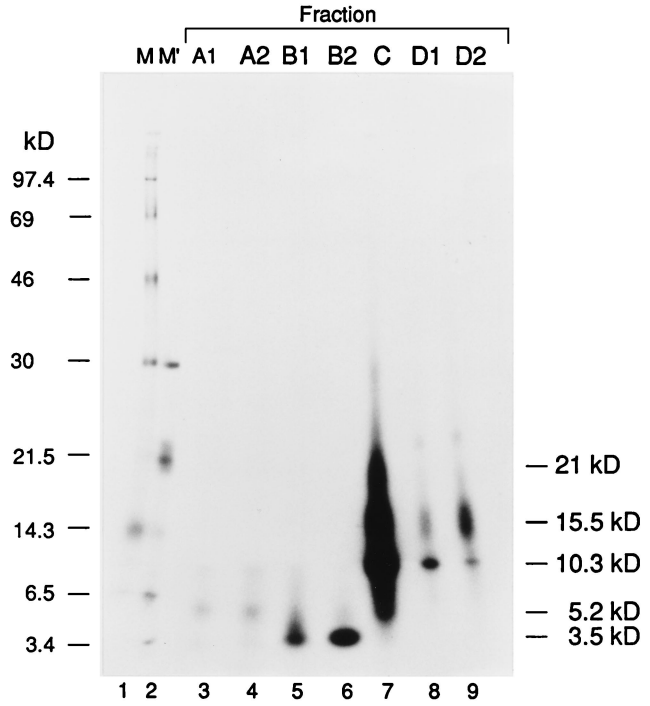

FIG. 2. Gel electrophoresis of HPLC fractions. Radiolabeled PKR was digested with cyanogen bromide and resolved by HPLC. Fractions were pooled as shown in Fig. 1D and analyzed by electrophoresis in a Tris-Tricine step gradient gel as in Fig. 1C. The positions and sizes of high- and low-molecular-weight markers (lanes M and M′) are indicated.

reactions after a phosphorylated residue (68). Peptide F8 is the only peptide in cyanogen bromide-digested PKR that is predicted to contain serine and/or threonine residues at cycles 3 and 6, namely, threonines 255 and 258 (i.e., no other peptide is predicted to have either a serine or a threonine residue at either position 3 or 6). The predicted molecular mass of peptide F8 (residues 253 to 264) is 1.6 kDa, so it is probably not visualized in gels such as that of Fig. 2. Consistent with these assignments, threonine was obtained upon phosphoamino acid analysis of fractions A1 and A2 (Fig. 3), but serine was obtained as well. The phosphoserine probably comes from a partial digestion product consisting of the conjoined peptide F7-F8 (Fig. 1A) which corresponds to the 5.2-kDa gel band

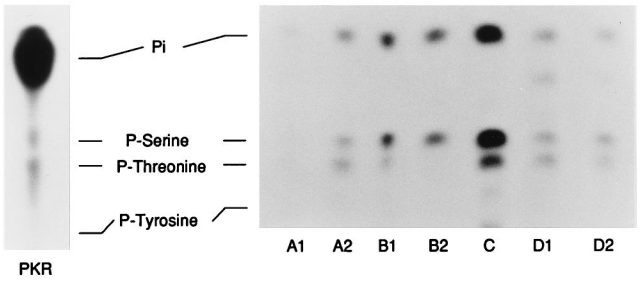

FIG. 3. Phosphoamino acid analysis of PKR and HPLC fractions. Autophosphorylated PKR and its peptides, derived by cyanogen bromide digestion and HPLC (as in Fig. 1D), were hydrolyzed with HCl. Soluble amino acids were resolved by electrophoresis on cellulose thin-layer chromatography plates and visualized by autoradiography. Standards were stained with ninhydrin. The positions of phosphoserine, phosphothreonine, phosphotyrosine, and P_i are indicated.

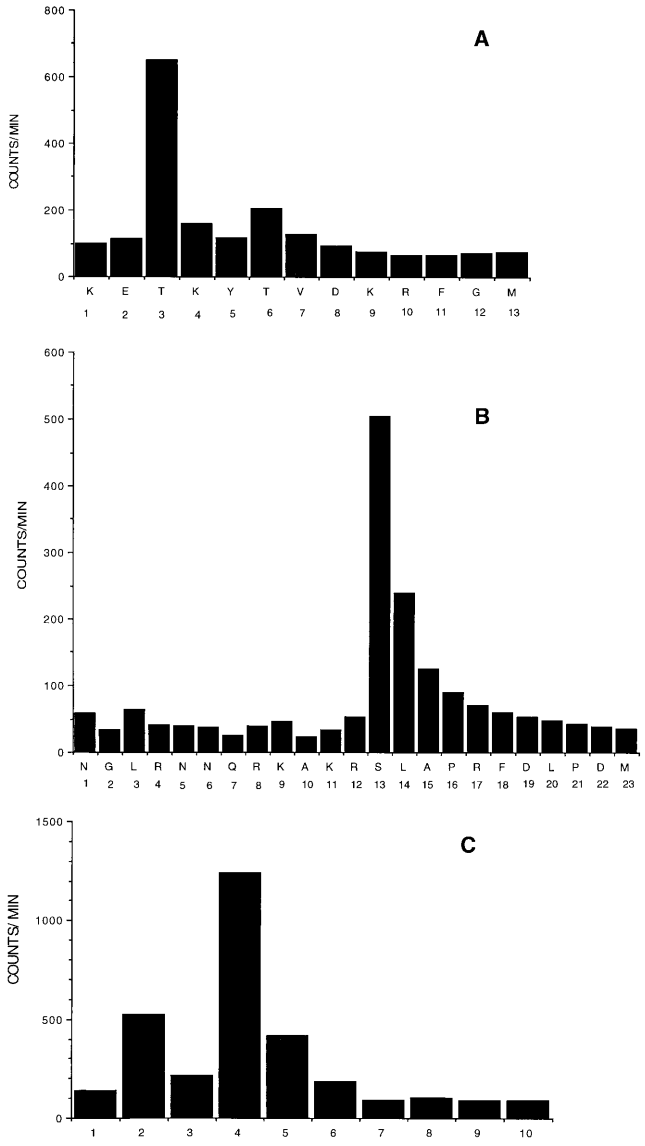

FIG. 4. Radioactive sequence analysis. Radioactive peaks, recovered from HPLC, were pooled as in Fig. 1D and sequenced with an automated protein sequencer. Amino acid derivatives were collected and assayed for radioactivity. (A and B) Radioactivity released from HPLC fractions A and B, respectively, at each Edman degradation cycle. The deduced amino acid sequence is displayed in single-letter code. (C) HPLC fraction B was digested further with the endoproteinase Lys-C and examined as described above.

(Fig. 2, lanes 3 and 4). This band is also present in fraction B1 (Fig. 2, lane 5). Evidence that F7 contains phosphoserine is summarized below.

Upon Edman degradation, fraction B gave a radioactive peak at cycle 13 (Fig. 4B). Peptide F7 is the only predicted peptide that contains a threonine or serine residue at position 13, namely, serine 242. Upon secondary digestion of fraction B material with the enzyme Lys-C, which cleaves after lysine residues, sequential Edman degradation gave radioactive peaks at cycles 2 and 4 (Fig. 4C). This confirms the serine 242 assignment, assuming that Lys-C gave rise to partial cleavage of F8 at lysines 238 and 240. Also consistent with this conclusion, serine was obtained as the predominant or sole phosphoamino acid (Fig. 3, B1 and B2, respectively). Furthermore,

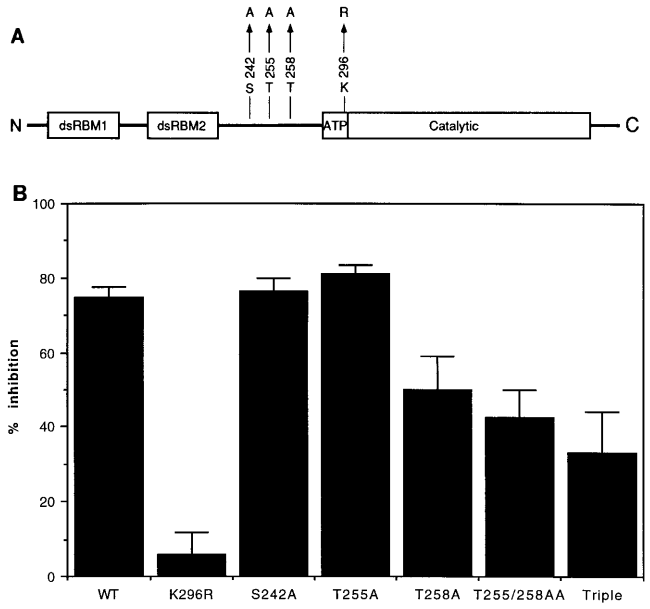

FIG. 5. Assay of PKR mutants in mammalian cells. (A) Diagram of PKR showing the locations of point mutations. Also depicted are the dsRNA-binding motifs dsRBM1 and dsRBM2 and the catalytic domain including the ATP-binding region. (B) Substitution mutants, carrying changes in individual amino acids or combinations, were expressed in monkey BSC-40 cells by using vaccinia virus-based vectors. The inhibition of protein synthesis due to PKR expression was measured by luciferase assay, and the results are presented in comparison with wild-type (WT) PKR and the catalytically inactive mutant K296R.

fraction B contained a prominent peptide with an apparent molecular mass of 3.5 kDa (Fig. 2, lanes 5 and 6), in reasonable agreement with the predicted molecular mass of peptide F7 (2.7 kDa), especially if retardation attributable to phosphorylation is taken into account. The presence of phosphothreonine in fraction B1, but not B2, correlates with the presence of the 5.2-kDa fusion peptide discussed above; its mobility is satisfactorily accounted for as the sum of 3.5 kDa for F7 and the predicted 1.6 kDa for F8.

These data from analysis of fractions A and B identify three closely spaced residues, serine 242, threonine 255, and threonine 258, as sites that are autophosphorylated when PKR is activated. The further analysis of fractions C and D will be described elsewhere (63a).

Effects of autophosphorylation site mutations in transfected mammalian cells. To assess the functional significance of the autophosphorylation sites, we examined the effects of mutations at these sites on PKR activity in monkey cells by using an infection-transfection assay (37). PKR expression vectors were constructed in which the autophosphorylation sites were substituted with residues that cannot accept a phosphate group. Serine 242 was changed to alanine, giving S242A; threonines 255 and 258 were changed to alanine individually or in combination, giving mutants T255A, T258A, and T255/258AA; and all three sites were changed to alanine, giving the Triple mutant, S242A/T255/258AA (Fig. 5A). The induction of PKR in transfected BSC-40 cells leads to inhibition of protein synthesized from a cotransfected luciferase reporter gene (36, 37). As shown in Fig. 5B, wild-type PKR reduced luciferase activity by about 75%, whereas the catalytically inactive mutant K296R did not elicit any significant inhibition. Mutation of serine 242 or threonine 255 had no detectable effect on the PKR-mediated inhibition of luciferase, but mutation of threonine 258 evinced sharply decreased activity. All of the mutants that contain the T258A mutation (T258A, T255/258AA, and Triple) were less active than the wild type, giving 50, 43, and 33% inhibition, respectively. Protein labeling experiments and immunoblots demonstrated that the mutant proteins were expressed at levels comparable to that of wild-type PKR (data not shown), so we concluded that threonine 258 is important for the activation of PKR in mammalian cells. The data of Fig. 5B, which are averaged from seven experiments, also suggest that the mutation of serine 242 and threonine 255 may exacerbate the effect of the T258A mutation, although the former mutations have no effect on their own.

TABLE 1. Yeast growth assay for kinase function[a]

PKR	Growth on:			
	SD	SGAL	SGAL–3-AT	SD–3-AT
Wild type	+	−	−	+
K296R	+	+	−	−
S242A	+	−	−	+
T255A	+	−	−	+
T258A	+	−	−	±
T255/258AA	+	−	−	±
Triple	+	−	−	±

[a] Yeast strain H1816 was transformed with wild-type or mutant PKR and grown at 30°C for 5 days in plates containing SD or SGAL medium with or without 3-AT. +, strong growth; ±, slow growth; −, no growth.

Effects of mutant PKR on cell growth and protein synthesis in *S. cerevisiae*. Interpretation of these findings might be complicated by the presence of endogenous PKR in BSC-40 cells. To circumvent this problem, we assayed the autophosphorylation site mutants in *S. cerevisiae* H1816, which lacks the endogenous eIF2 kinase GCN2. The cDNA encoding wild-type or mutant PKR was placed under the control of the galactose-inducible GAL1-CYC1 promoter, and cell growth was measured on selective media, allowing the phenotypes of different PKR mutants to be scored (58). As shown in Table 1, all cells containing either mutant or wild-type kinase grew on glucose-containing minimal medium SD. High-level expression of wild-type PKR in galactose-containing medium SGAL results in a high cellular level of phosphorylated eIF2 and severely decreases cell growth (9, 11, 58). The catalytically inactive mutant K296R grows well on SGAL, as expected. When histidine biosynthesis is blocked by 3-aminotriazole (3-AT), growth becomes dependent on a moderate degree of phosphorylation of eIF2 that elicits increased synthesis of GCN4 and its target genes in the histidine biosynthetic pathway; consequently, wild-type PKR permits growth on SD–3-AT but the K296R mutant enzyme does not. The excessive eIF2 phosphorylation that occurs when PKR is induced by galactose also suppresses growth, by blocking overall protein synthesis, so neither strains transformed with wild-type PKR nor those transformed with its K296R mutant grow in SGAL–3-AT medium (Table 1).

Like cells transformed with wild-type PKR, cells containing either the S242A or the T255A mutant form did not grow on SGAL medium but grew well on SD–3-AT medium, indicating that these mutations do not detectably alter PKR activity. On the other hand, cells containing the T258A mutation, either singly or in combination with other mutations (in T255/258AA and Triple), grew modestly on SD–3-AT medium and poorly on SGAL medium. This behavior signifies a reduction in PKR activity, confirming that threonine 258 is important for PKR activity.

Another measure of PKR function depends on the observation that the wild-type kinase down-regulates its own synthesis

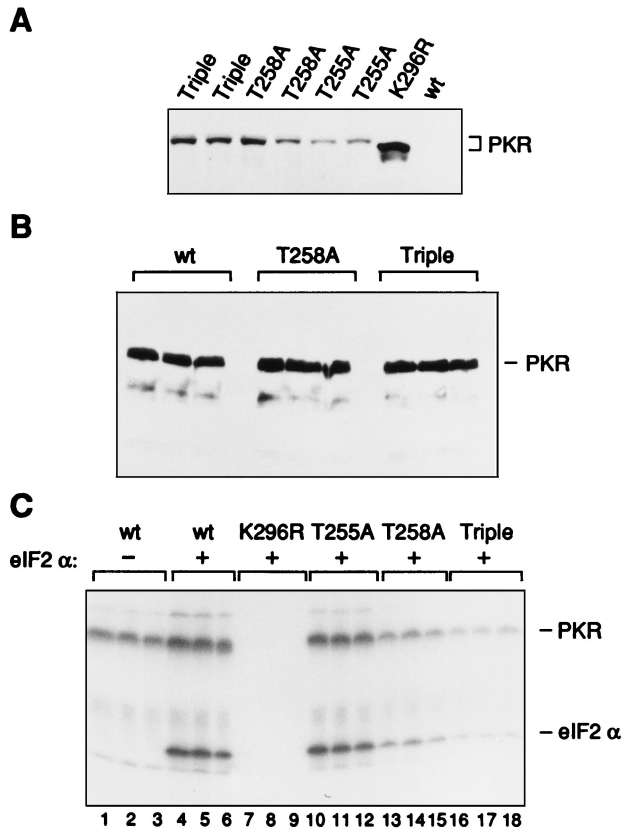

FIG. 6. Yeast assays of PKR function. (A) Expression of wild-type (wt) PKR and mutant PKR in yeast strain H1816 (carrying a wild-type eIF2α gene). Protein extracts were resolved in a polyacrylamide-SDS gel, blotted, and probed with a anti-PKR antibody. Duplicate samples of protein extracts were loaded for cells transformed with the Triple, T258A, and T255A mutants, and single samples were loaded for K296R and the wild type. (B) Expression of wild-type and mutant PKR in yeast strain H1817, which carries a mutant eIF2α gene. Extracts were examined as described for panel A. (C) Autophosphorylation of wild-type PKR and mutant PKR and phosphorylation of eIF2α in vitro. Kinase assays were conducted with PKR isolated on antibody-coated beads in the presence (+) or absence (−) of eIF2. For panels B and C, triplicate samples were loaded on the gels. Detection was by excited chemiluminescence (A and B) and autoradiography (C).

(2, 36, 58, 66). This can be monitored in extracts of PKR-transformed H1816 yeast cells by immunoblot analysis (Fig. 6A). As shown previously by Romano et al. (58), wild-type PKR was expressed at a low level (barely visible in Fig. 6A) whereas the catalytically inactive K296R mutant was expressed at a high level in this strain. Each of the mutant proteins was expressed at a higher level than the wild-type protein but at a lower level than the K296R protein. The T255A mutant PKR accumulated to a lesser extent than mutants containing the threonine 258 mutation (T258A and Triple). Since accumulation is inversely related to kinase activity, these results imply that the T258A mutation is more deleterious than the T255A mutation to PKR function, but both threonine residues play some role. The S242A mutation has no discernible effect on PKR accumulation (data not shown). In strain H1817, which carries a mutated form of the eIF2α subunit that cannot be phosphorylated, the mutant and wild-type kinases all accumulated to similar levels (Fig. 6B), demonstrating that the changes in PKR accumulation seen in Fig. 6A are mediated by phosphorylation of this initiation factor.

Effects of mutations on PKR activity in vitro. Mutant PKR and wild-type PKR were expressed in the 1817 yeast strain, which permits the kinase to accumulate to high levels, and protein extracts were prepared. The enzyme was captured on antibody-loaded beads, and kinase assays were used to measure directly the effects of the mutations on the ability of PKR to autophosphorylate and to phosphorylate eIF2. Wild-type PKR was efficiently autophosphorylated, both on its own (Fig. 6C, lanes 1 to 3) and in the presence of eIF2 (lanes 4 to 6), when it also phosphorylated the α subunit of eIF2. The K296R mutant was completely inactive, both for autophosphorylation and for phosphorylation of eIF2α (lanes 7 to 9). The mutant T255A was nearly as active as the wild-type kinase (lanes 10 to 12), while mutants containing the T258A mutation (T258A and Triple, lanes 13 to 18) were substantially impaired for both autophosphorylation and phosphorylation of eIF2α. With respect to PKR autophosphorylation, similar results were obtained in assays conducted in the absence of added eIF2. Quantitation of the assays showed that the T258A and Triple mutants were 1.6- and 2.5-fold less active than wild-type PKR for autophosphorylation and 2- and 3.8-fold less active than the wild-type enzyme for eIF2 phosphorylation. The lower activity of the Triple mutant relative to the T258A mutant also suggests that the removal of additional potential phosphorylation sites at serine 242 and threonine 255 may exacerbate the T258A mutation although the mutations at these sites may not exert significant effects by themselves.

DISCUSSION

PKR is found in most mammalian cells at low levels and in a latent state. Expression of the enzyme is induced by interferon, and it is activated upon viral infection, most probably by viral dsRNA (39). Activation is accompanied by autophosphorylation on several serine and threonine residues (18, 33). Following activation, the enzyme is able to phosphorylate its substrates, including eIF2 and IκB. In this study, we investigated the sites on PKR that are autophosphorylated during activation by dsRNA in vitro and evaluated the roles played by these sites in kinase activation. We found a major region of phosphorylation in the enzyme's RNA-binding domain (63a) and three sites of autophosphorylation in its third basic region. One of these, threonine 258, was found to be important for kinase activity in a vaccinia virus-based assay, a yeast growth assay, and kinase assays conducted in vitro. Mutation of this threonine to a nonphosphorylatable residue decreased the enzymes' activity. Moreover, the presence of neighboring residues that can be phosphorylated, namely, serine 242 and especially threonine 255, appeared to increase the activity of the enzyme. It is noteworthy that threonine 258 is conserved between mouse PKR and human PKR, although much of the third basic region, including its basic character, is not (15). Serine 242 and threonine 255, present in the human enzyme, are not conserved, but their ancillary roles may be taken by other phosphate-accepting residues in the corresponding region of the murine kinase.

The binding of dsRNA to PKR is thought to lead to a conformational change which permits PKR to autophosphorylate, thereby switching its substrate specificity and committing it to phosphorylate external substrates (30, 38, 53). Accordingly, the RNA-binding domain is required for activation of PKR (2, 15, 21, 58). However, the RNA-binding domain was dispensable for activation of the enzyme when an endogenous eIF2 kinase was present (36), presumably because the defective kinase is phosphorylated in *trans*. Such *trans* phosphorylation of a catalytically inactive PKR mutant by the wild-type

enzyme has been demonstrated in vitro (66). In this circumstance, the third basic region was indispensable for kinase function (36). Furthermore, a deletion mutant lacking residues 233 to 271 was poorly phosphorylated. In view of the data presented here, we propose that activation of the kinase occurs through intermolecular phosphorylation of sites in the third basic region and that threonine 258 is a critical autophosphorylation site. Reminiscent of some receptor tyrosine kinases which contain redundant autophosphorylation sites (13, 47), the observation that mutation of threonine 258 does not abolish the activity and phosphorylation of PKR may imply the existence of additional critical sites in PKR.

Many kinases are regulated, either positively or negatively, by phosphorylation. Several are phosphorylated by other kinases, and kinase cascades are becoming increasingly familiar. Others are activated through autophosphorylation. Protein kinase C is more active when autophosphorylated on serine and threonine residues (16, 46), and autophosphorylation on tyrosine residues increases the activity of $pp60^{c\text{-}src}$ (28, 52), the insulin receptor (13, 23), and $p130^{gag\text{-}fps}$ (67). Although the sites vary, activation by phosphorylation in the catalytic domain between subdomains VII and VIII is common. Our analysis revealed no such sites in PKR, however. Instead, the threonine 258 site that has been shown to play a role in enzyme activation is located between the catalytic domain and the RNA-binding domain, in a region that may serve as a "hinge." Its phosphorylation would render the region more negatively charged and might open or close the hinge, altering the interaction between the regulatory and catalytic domains in a fashion similar to that suggested previously for PKC (16). Although the precise effects of autophosphorylation will be appreciated only in light of an understanding of the enzyme's three-dimensional structure, our results emphasize that the third basic region is important for PKR activation and indicate that it defines an autoregulatory domain critical for converting the kinase to a fully active mode.

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REFERENCES

1. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.** 1991. Current protocols in molecular biology. John Wiley & Sons, Inc., New York.
2. **Barber, G. N., S. Edelhoff, M. G. Katze, and C. M. Disteche.** 1993. Chromosomal assignment of the interferon-inducible double-stranded RNA-dependent protein kinase (PRKR) to human chromosome 2p21-p22 and mouse chromosome 17 E2. Genomics **16:**765–767.
3. **Berry, M. J., G. S. Knutson, S. R. Lasky, S. M. Munemitsu, and C. E. Samuel.** 1985. Purification and substrate specificities of the double-stranded RNA-dependent protein kinase from untreated and interferon-treated mouse fibroblasts. J. Biol. Chem. **260:**11240–11247.
4. **Boyle, W. J., P. van der Geer, and T. Hunter.** 1991. Phosphopeptide mapping and phosphoamino acid analysis by two-dimensional separation on thin-layer cellulose plates. Methods Enzymol. **201:**110–149.
5. **Brand, S. R., R. Kobayashi, and M. B. Mathews.** The Tat protein of human immunodeficiency virus type 1 is a substrate and inhibitor of the interferon-induced, virus-activated protein kinase, PKR. Submitted for publication.
6. **Brasier, A. R., J. E. Tate, and J. F. Habener.** 1989. Optimized use of the firefly luciferase assay as a reporter gene in mammalian cell lines. BioTechniques **7:**1116–1122.
7. **Bycroft, M., S. Grünert, A. G. Murzin, M. Proctor, and D. St Johnston.** 1995. NMR solution structure of a dsRNA binding domain from *Drosophila* staufen protein reveals homology to the N-terminal domain of ribosomal protein S5. EMBO J. **14:**3563–3571.
8. **Chen, J.-J., M. S. Throop, L. Gehrke, I. Kuo, J. K. Pal, M. Brodsky, and I. M. London.** 1991. Cloning of the cDNA of the heme-regulated eukaryotic initiation factor 2a (eIF-2a) kinase of rabbit reticulocytes: homology to yeast GCN2 protein kinase and human double-stranded-RNA-dependent eIF-2a kinase. Proc. Natl. Acad. Sci. USA **88:**7729–7733.
9. **Chong, K. L., L. Feng, K. Schappert, E. Meurs, T. F. Donahue, J. D. Friesen, A. G. Hovanessian, and B. R. G. Williams.** 1992. Human p68 kinase exhibits growth suppression in yeast and homology to the translational regulator *GCN2*. EMBO J. **11:**1553–1562.
10. **Clemens, M. J.** 1996. Protein kinases that phosphorylate eIF2 and eIF2B, and their role in eukaryotic cell translational control, p. 139–172. *In* J. W. B. Hershey, M. B. Mathews, and N. Sonenberg (ed.), Translational control. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
11. **Dever, T. E., J. Chen, G. N. Barber, A. M. Cigan, L. Feng, T. F. Donahue, I. M. London, M. G. Katze, and A. G. Hinnebusch.** 1993. Mammalian eukaryotic initiation factor 2a kinases functionally substitute for GCN2 protein kinase in the GCN4 translational control mechanism of yeast. Proc. Natl. Acad. Sci. USA **90:**4616–4620.
12. **Dever, T. E., L. Feng, R. C. Wek, A. M. Cigan, T. F. Donahue, and A. G. Hinnebusch.** 1992. Phosphorylation of initiation factor 2a by protein kinase GCN2 mediates gene-specific translational control of *GCN4* in yeast. Cell **68:**585–596.
13. **Ellis, L., E. Clauser, D. O. Morgan, M. Edery, R. A. Roth, and W. J. Rutter.** 1986. Replacement of insulin receptor tyrosine residues 1162 and 1163 compromises insulin-stimulated kinase activity and uptake of 2-deoxyglucose. Cell **45:**721–732.
14. **Farrell, P. J., K. Balkow, T. Hunt, R. J. Jackson, and H. Trachsel.** 1977. Phosphorylation of initiation factor eIF-2 and the control of reticulocyte protein synthesis. Cell **11:**187–200.
15. **Feng, G.-S., K. Chong, A. Kumar, and B. R. G. Williams.** 1992. Identification of double-stranded RNA-binding domains in the interferon-induced double-stranded RNA-activated p68 kinase. Proc. Natl. Acad. Sci. USA **89:**5447–5451.
16. **Flint, A. J., R. D. Paladini, and D. E. J. Koshland.** 1990. Autophosphorylation of protein kinase C at three separated regions of its primary sequence. Science **249:**408–411.
17. **Galabru, J., and A. Hovanessian.** 1985. Two interferon-induced proteins are involved in the protein kinase complex dependent on double-stranded RNA. Cell **43:**685–694.
18. **Galabru, J., and A. Hovanessian.** 1987. Autophosphorylation of the protein kinase dependent on double-stranded RNA. J. Biol. Chem. **262:**15538–15544.
19. **Galabru, J., B. Krust, and A. G. Hovanessian.** 1984. Interferon-mediated protein kinase activity in different fractions of mouse L-929 cells. J. Interferon Res. **4:**469–480.
20. **Green, S. R., L. Manche, and M. B. Mathews.** 1995. Two functionally distinct RNA-binding motifs in the regulatory domain of the protein kinase DAI. Mol. Cell. Biol. **15:**358–364.
21. **Green, S. R., and M. B. Mathews.** 1992. Two RNA binding motifs in the double-stranded RNA activated protein kinase, DAI. Genes Dev. **6:**2478–2490.
22. **Hanks, S. K., A. M. Quinn, and T. Hunter.** 1988. The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. Science **241:**42–52.
23. **Herrera, R., and O. M. Rosen.** 1986. Autophosphorylation of the insulin receptor *in vitro*. J. Biol. Chem. **261:**11980–11985.
24. **Hinnebusch, A. G.** 1990. Involvement of an initiation factor and protein phosphorylation in translational control of *GCN4* mRNA. Trends Biochem. Sci. **15:**148–152.
25. **Hinnebusch, A. G.** 1996. Translational control of GCN4: gene-specific regulation by phosphorylation of eIF2, p. 199–244. *In* J. W. B. Hershey, M. B. Mathews, and N. Sonenberg (ed.), Translational control. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
26. **Katze, M. G.** 1996. Translational control in cells infected with influenza virus and reovirus, p. 607–630. *In* J. W. B. Hershey, M. B. Mathews, and N. Sonenberg (ed.), Translational control. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
27. **Katze, M. G., M. Wambach, M.-L. Wong, M. Garfinkel, E. Meurs, K. Chong, B. R. G. Williams, A. G. Hovanessian, and G. N. Barber.** 1991. 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. Mol. Cell. Biol. **11:**5497–5505.
28. **Kmiecik, T. E., and D. Shalloway.** 1987. Activation and suppression of $pp60^{c\text{-}src}$ transforming ability by mutation of its primary sites of tyrosine phosphorylation. Cell **49:**65–73.
29. **Koromilas, A. E., S. Roy, G. N. Barber, M. G. Katze, and N. Sonenberg.** 1992. Malignant transformation by a mutant of the IFN-inducible dsRNA-dependent protein kinase. Science **257:**1685–1689.
30. **Kostura, M., and M. B. Mathews.** 1989. Purification and activation of the double-stranded RNA-dependent eIF-2 kinase DAI. Mol. Cell. Biol. **9:**1576–1586.
31. **Kumar, A., J. Haque, J. Lacoste, J. Hiscott, and B. R. G. Williams.** 1994. Double-stranded RNA-dependent protein kinase activates transcription factor NF-κB by phosphorylating IκB. Proc. Natl. Acad. Sci. USA **91:**6288–6292.

32. **Laemmli, E. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
33. **Lasky, S. R., B. L. Jacobs, and C. E. Samuel.** 1982. Characterization of the sites of phosphorylation in the interferon-induced phosphoprotein P1 from mouse fibroblasts: evidence for two forms of P1. J. Biol. Chem. **257:**11087–11093.
34. **Laurent, A. G., B. Krust, J. Galabru, J. Svab, and A. G. Hovanessian.** 1985. Monoclonal antibodies to an interferon-induced Mr 68,000 protein and their use for the detection of double-stranded RNA-dependent protein kinase in human cells. Proc. Natl. Acad. Sci. USA **82:**4341–4345.
35. **Lee, S. B., and M. Esteban.** 1994. The interferon-induced double-stranded RNA-activated protein kinase induces apoptosis. Virology **199:**491–496.
36. **Lee, S. B., S. R. Green, M. B. Mathews, and M. Esteban.** 1994. Activation of the double-stranded RNA-activated human protein kinase *in vivo* in the absence of its dsRNA binding domain. Proc. Natl. Acad. Sci. USA **91:**10551–10555.
37. **Lee, S. B., Z. Melkova, W. Yan, B. R. G. Williams, A. G. Hovanessian, and M. Esteban.** 1993. The interferon-induced double-stranded RNA-activated human p68 protein kinase potently inhibits protein synthesis in cultured cells. Virology **192:**380–385.
38. **Manche, L., S. R. Green, C. Schmedt, and M. B. Mathews.** 1992. Interactions between double-stranded RNA regulators and the protein kinase DAI. Mol. Cell. Biol. **12:**5238–5248.
39. **Maran, A., and M. B. Mathews.** 1988. Characterization of the double-stranded RNA implicated in the inhibition of protein synthesis in cells infected with a mutant adenovirus defective for VA RNA_1. Virology **164:**106–113.
40. **Mathews, M. B.** 1996. Interactions between viruses and the cellular machinery for protein synthesis, p. 505–548. *In* J. W. B. Hershey, M. B. Mathews, and N. Sonenberg (ed.), Translational control. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
41. **Mathews, M. B., and T. Shenk.** 1991. Adenovirus virus-associated RNA and translational control. J. Virol. **65:**5657–5662.
42. **McMillan, N. A. J., R. F. Chun, D. P. Siderovski, J. Galabru, W. M. Toone, C. E. Samuel, T. W. Mak, A. G. Hovanessian, K. Jeang, and B. R. G. Williams.** 1995. HIV-1 Tat directly interacts with the interferon-induced, double-stranded RNA-dependent kinase, PKR. Virology **213:**413–424.
43. **Merrick, W. C., and J. W. B. Hershey.** 1996. The pathway and mechanism of eukaryotic protein synthesis, p. 31–69. *In* J. W. B. Hershey, M. B. Mathews, and N. Sonenberg (ed.), Translational control. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
44. **Meurs, E., K. Chong, J. Galabru, N. S. B. Thomas, I. M. Kerr, B. R. G. Williams, and A. G. Hovanessian.** 1990. Molecular cloning and characterization of the human double-stranded RNA-activated protein kinase induced by interferon. Cell **62:**379–390.
45. **Meurs, E. F., J. Galabru, G. N. Barber, M. G. Katze, and A. G. Hovanessian.** 1993. Tumor suppressor function of the interferon-induced double-stranded RNA-activated protein kinase. Proc. Natl. Acad. Sci. USA **90:**232–236.
46. **Mochly-Rosen, D., and D. E. J. Koshland.** 1987. Domain structure and phosphorylation of protein kinase C. J. Biol. Chem. **262:**2291–2297.
47. **Mohammadi, M., I. Dikic, A. Sorokin, W. H. Burgess, M. Jaye, and J. Schlessinger.** 1996. Identification of six novel autophosphorylation sites on fibroblast growth factor receptor 1 and elucidation of their importance in receptor activation and signal transduction. Mol. Cell. Biol. **16:**977–989.
48. **Mundschau, L. J., and D. V. Faller.** 1995. Platelet-derived growth factor signal transduction through the interferon-inducible kinase PKR. Immediate early gene induction. J. Biol. Chem. **270:**3100–3106.
49. **Offermann, M. K., J. Zimring, K. H. Mellits, M. K. Hagan, R. Shaw, R. M. Medford, M. B. Mathews, S. Goodbourn, and R. Jagus.** 1995. Activation of the double-stranded-RNA-activated protein kinase and induction of vascular cell adhesion molecule-1 by poly(I) × poly(C) in endothelial cells. Eur. J. Biochem. **232:**28–36.
50. **Patel, R. C., and G. C. Sen.** 1992. Identification of the double-stranded RNA-binding domain of the human interferon-inducible protein kinase. J. Biol. Chem. **267:**7671–7676.
51. **Petryshyn, R., J.-J. Chen, and I. M. London.** 1984. Growth-related expression of a double-stranded RNA-dependent protein kinase in 3T3 cells. J. Biol. Chem. **259:**14736–14742.
52. **Piwnica-Worms, H., K. B. Saunders, T. M. Roberts, A. E. Smith, and S. H. Cheng.** 1987. Tyrosine phosphorylation regulates the biochemical and biological properties of $pp60^{c\text{-}src}$. Cell **49:**75–82.
53. **Proud, C. G.** 1995. PKR: a new name and new roles. Trends Biochem. Sci. **20:**241–246.
54. **Proud, C. G., D. R. Colthurst, S. Ferrari, and L. A. Pinna.** 1990. The substrate specificity of protein kinases which phosphorylate the α subunit of eukaryotic initiation factor 2. Eur. J. Biochem. **195:**771–779.
55. **Rice, A. R., M. Kostura, and M. B. Mathews.** 1989. Identification of a 90 KD polypeptide which associates with adenovirus VA RNA_1 and is phosphorylated by the double-stranded RNA dependent protein kinase. J. Biol. Chem. **264:**20632–20637.
56. **Rodriguez, J. F., D. Rodriguez, J. Rodriguez, E. B. McGowan, and M. Esteban.** 1988. Expression of the firefly luciferase gene in vaccinia virus: a highly sensitive gene marker to follow virus dissemination in tissues of infected animals. Proc. Natl. Acad. Sci. USA **85:**1667–1671.
57. **Rodriguez, J. F., and G. L. Smith.** 1990. Inducible gene expression from vaccinia virus vectors. Virology **177:**239–250.
58. **Romano, P. R., S. R. Green, G. N. Barber, M. B. Mathews, and A. G. Hinnebusch.** 1995. Structural requirements for double-stranded RNA binding, dimerization, and activation of the human eIF-2α kinase DAI in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **15:**365–378.
59. **Russo, G. L., M. T. Vandenberg, I. Yu, Y.-S. Bae, B. R. Franza, Jr., and D. R. Marshak.** 1992. Casein kinase II phosphorylates $p34^{cdc2}$ kinase in G1 phase of the HeLa cell division cycle. J. Biol. Chem. **267:**20317–20325.
60. **Samuel, C. E.** 1979. Mechanism of interferon action: phosphorylation of protein synthesis initiation factor eIF-2 in interferon-treated human cells by a ribosome-associated kinase processing site specificity similar to hemin-regulated rabbit reticulocyte kinase. Proc. Natl. Acad. Sci. USA **76:**600–604.
61. **Schägger, H., and G. von Jagow.** 1987. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal. Biochem. **166:**368–379.
62. **Schneider, R. J.** 1996. Adenovirus and vaccinia virus translational control, p. 575–605. *In* J. W. B. Hershey, M. B. Mathews, and N. Sonenberg (ed.), Translational conrol. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
63. **St Johnston, D., N. H. Brown, J. G. Gall, and M. Jantsch.** 1992. A conserved double-stranded RNA-binding domain. Proc. Natl. Acad. Sci. USA **89:**10979–10983.

63a. **Taylor, D. R., and M. B. Mathews.** Unpublished data.

64. **Thomis, D. C., J. P. Doohan, and C. E. Samuel.** 1992. Mechanism of interferon action: cDNA structure, expression, and regulation of the interferon-induced, RNA-dependent P1/eIF-2a protein kinase from human cells. Virology **188:**33–46.
65. **Thomis, D. C., and C. E. Samuel.** 1993. Mechanism of interferon action: evidence of intermolecular autophosphorylation and autoactivation of the interferon-induced, RNA-dependent protein kinase PKR. J. Virol. **67:**7695–7700.
66. **Thomis, D. C., and C. E. Samuel.** 1995. Mechanism of interferon action: characterization of the intermolecular autophosphorylation of PKR, the interferon-inducible, RNA-dependent protein kinase. J. Virol. **69:**5195–5198.
67. **Weinmaster, G., and T. Pawson.** 1986. Protein kinase activity of FSV (fujinami sarcoma virus) $P130^{gag\text{-}fps}$ shows a strict specificity for tyrosine residues. J. Biol. Chem. **261:**328–333.
68. **Wettenhall, R. E. H., R. H. Aebersold, and L. E. Hood.** 1991. Solid-phase sequencing of ^{32}P-labeled phosphopeptides at picomole and subpicomole levels. Methods Enzymol. **201:**186–199.
69. **Yang, Y., F. L. Reis, J. Pavlovic, A. Aguzzi, R. Schafer, A. Kumar, B. R. G. Williams, M. Aguet, and C. Weissmann.** 1995. Deficient signaling in mice devoid of double-stranded RNA-dependent protein kinase. EMBO J. **14:**6095–6106.
70. **Zoller, M. J., and M. Smith.** 1984. Oligonucleotide-directed mutagenesis: a simple method using two oligonucleotide primers and a single-stranded DNA template. DNA **3:**479–488.