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Hepatitis C Virus Envelope Protein E2 Does Not Inhibit PKR by Simple Competition with Autophosphorylation Sites in the RNA-Binding Domain

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Double-stranded-RNA (dsRNA)-dependent protein kinase PKR is induced by interferon and activated upon autophosphorylation. We previously identified four autophosphorylated amino acids and elucidated their participation in PKR activation. Three of these sites are in the central region of the protein, and one is in the kinase domain. Here we describe the identification of four additional autophosphorylated amino acids in the spacer region that separates the two dsRNA-binding motifs in the RNA-binding domain. Eight amino acids, including these autophosphorylation sites, are duplicated in hepatitis C virus (HCV) envelope protein E2. This region of E2 is required for its inhibition of PKR although the mechanism of inhibition is not known. Replacement of all four of these residues in PKR with alanines did not dramatically affect kinase activity in vitro or in yeast *Saccharomyces cerevisiae*. However, when coupled with mutations of serine 242 and threonines 255 and 258 in the central region, these mutations increased PKR protein expression in mammalian cells, consistent with diminished kinase activity. A synthetic peptide corresponding to this region of PKR was phosphorylated in vitro by PKR, but phosphorylation was strongly inhibited after PKR was preincubated with HCV E2. Another synthetic peptide, corresponding to the central region of PKR and containing serine 242, was also phosphorylated by active PKR, but E2 did not inhibit this peptide as efficiently. Neither of the PKR peptides was able to disrupt the HCV E2-PKR interaction. Taken together, these results show that PKR is autophosphorylated on serine 83 and threonines 88, 89, and 90, that this autophosphorylation may enhance kinase activation, and that the inhibition of PKR by HCV E2 is not solely due to duplication of and competition with these autophosphorylation sites.

Hepatitis C virus (HCV) is an emerging pathogen of increasing medical significance. As many as 2% of the world population may now be infected with HCV, and possibly 90% of these individuals will become chronically infected (14). The only approved therapies for HCV are alpha interferon (IFN-α) monotherapy or combination therapy with ribavirin. IFN is only effective in about 10% of patients, but in these patients a sustained response is accompanied by clearance of viral RNA. Genotypic variants display different rates of response to IFN-α, and these genotypes are characterized by mutations that may be responsible for IFN-α resistance or sensitivity (12). Two HCV proteins that have been implicated in IFN resistance through inhibition of the IFN-α-induced, double-stranded-RNA (dsRNA)-activated protein kinase (PKR) are NS5A and E2 (19, 52). The nonstructural protein, NS5A, contains mutations in a region known as the IFN-α-sensitivity-determining region in Japanese isolates; these mutations result in a lack of PKR binding to NS5A and thus IFN-α sensitivity (for a review, see reference 50). E2 contains a region of identity with PKR and its substrate, eIF2α, termed the PKR-eIF2α phosphorylation site homology domain or PePHD; although the exact mechanism is unknown, this region is required for PKR inhibition (52).

PKR is a serine/threonine kinase found in cells in a latent state. It plays a part in cellular antiviral defense as well as in apoptosis, signal transduction, and transformation (reviewed in references 10, 24, 38, and 56). PKR is activated by autophosphorylation upon binding to its regulator, dsRNA, and similar molecules (24, 38). Activation apparently occurs by an intermolecular autophosphorylation reaction (26, 55), permitting the enzyme to phosphorylate its substrates. Best known of these is translational initiation factor eIF2, which is phosphorylated on serine 51 of its α subunit (15, 39). Phosphorylation of eIF2α mediates a number of cellular processes, most notably the shutoff of protein synthesis (reviewed in references 10, 24, and 56). PKR also phosphorylates several cellular and viral proteins, including the human immunodeficiency virus transactivator protein, Tat (5, 34), and 90-kDa proteins from rabbit reticulocytes (40) and human cells (8, 53; L. Parker, I. Fierro-

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Monti, and M. B. Mathews, unpublished data). PKR mediates the phosphorylation of IκB, the inhibitor of NF-κB (27, 32, 36), in response to dsRNA although the role of PKR in this pathway is unclear (4). Phosphorylation of IκB enables NF-κB to translocate to the nucleus, and both NIK and IKK are components in this pathway (reviewed in reference 2). Transgenic mice devoid of functional PKR are unresponsive to activators of NF-κB, further supporting the role of PKR in signal transduction pathways (28). The roles of the other phosphorylation events are as yet unknown.

In addition to PKR, three other kinases can regulate protein synthesis through phosphorylation of eIF2 (7, 22, 44). In reticulocytes, the heme-regulated inhibitor, HRI, controls protein synthesis levels in response to the availability of heme (7). The yeast *Saccharomyces cerevisiae* regulates amino acid biosynthesis through translational derepression of transcriptional activator GCN4 via GCN2 kinase, and a homologue of the yeast enzyme has recently been isolated in *Drosophila melanogaster* (22). Recently described eIF2 kinase PERK or PEK is responsible for reduced protein synthesis during endoplasmic reticulum stress (44).

PKR has homology with these kinases in its catalytic domain, which occupies the C-terminal half of the protein. The N-terminal one-third of PKR appears to function as its regulatory domain and has homology with other dsRNA-binding proteins (6, 21, 25, 49). The RNA-binding domain (RBD) of PKR consists of two repeats of a 65- to 68-amino-acid-long motif, the dsRNA-binding motif (dsRBM); these repeats are rich in basic amino acids (17). The repeats, dsRBM-1 and dsRBM-2 (amino acids 11 to 77 and 101 to 167, respectively), are separated by a short unstructured spacer (6, 16, 20, 21, 23, 35, 37, 43, 49). The spacer region is important for RNA binding, as a mutant with a large deletion within this region fails to bind RNA efficiently (21), possibly because of structural constraints between dsRBM-1 and dsRBM-2. Separating the RBD from the kinase domain of PKR is the central region (amino acids 233 to 268), which is also rich in basic amino acids but which is not homologous to the dsRBMs.

Four sites of autophosphorylation in PKR have been described (42, 51), out of a total of 14 sites that may be phosphorylated in vivo in yeast (58). Three of these sites are located in the central region and participate in activation of the kinase. These sites were identified through peptide mapping and sequencing of PKR phosphorylated during activation by dsRNA in vitro. The fourth and possibly fifth sites of autophosphorylation were identified through mass spectrometry and genetic analysis (42). These sites are located in the activation loop within the kinase domain and play a role in kinase activation. Here we describe the identification of four prominent autophosphorylation sites that are located in the spacer region between the two dsRBMs in the RBD. Mutagenesis of these four autophosphorylation sites did not affect enzyme activation in yeast or in vitro or the binding of dsRNA but led to decreased PKR expression in mammalian cells when combined with mutations of other autophosphorylation sites in the central region of PKR. These results suggest that phosphorylation of serine 83 and threonines 88, 89, and 90 contribute to full activation of the kinase. Eight amino acids in the sequence containing these phosphorylation sites are identical to residues in the HCV E2 protein. HCV E2 inhibited the phosphorylation of PKR peptides, particularly that of the peptide that corresponds to the homologous region in the spacer. The PKR peptides did not effectively compete with HCV E2 for binding to PKR, however, suggesting that the inhibition of PKR is not due to simple competition at these autophosphorylation sites.

MATERIALS AND METHODS

Generation of radiolabeled peptides. PKR, purified to the mono-S stage from IFN-α-treated 293 cells (26), was activated in vitro in the presence of reovirus dsRNA (provided by A. Shatkin) and [γ-^{32}P]ATP under conditions described previously (51). Radiolabeled PKR was immunoprecipitated with a polyclonal antibody (21) and was eluted from protein A-Sepharose beads with formic acid, digested with cyanogen bromide (CNBr) as described previously (51), and lyophilized. The CNBr digestion products were separated by high-performance liquid chromatography (HPLC) (51), fractions were collected and counted by Cerenkov radiation, and the radioactive peaks were pooled. Radiolabeled peptides were resolved in Tris-Tricine gels (47); the gels were fixed, dried, and exposed to film for autoradiography.

Secondary peptidase digestion. Secondary digestion of radiolabeled PKR peptides was performed with the following enzymes: endoproteinases Lys-C, Asp-N, and Arg-C (sequencing grade; Boehringer Mannheim); trypsin (tolylsulfonyl phenylalanyl chlomethyl ketone treated; Sigma); chymotrypsin (TLCK [*N*α-*p*-tosyl-L-lysine chloromethyl ketone] treated; Sigma); and *Staphylococcus aureus* V8 (Sigma). Digestion with Arg-C was conducted in accordance with the manufacturer's specifications, and digestion with Lys-C, Asp-N, trypsin, and chymotrypsin was performed as described previously for Lys-C (51).

Radioactive sequence analysis. Secondary digests were lyophilized, resuspended in 60% acetonitrile, and coupled to arylamine-derivatized polyvinylidene difluoride (PVDF) membranes (Sequalon AA; Millipore) with carbodiimide. PVDF-bound peptides were subjected to repetitive Edman degradation reactions on a protein sequencer (Applied Biosystems; 473A) (45), and radioactivity was monitored as described above.

Kinase assays. For peptide phosphorylation kinetics and phosphoamino acid analysis, mono S-purified PKR (26) was added to a kinase reaction mixture (51) and the mixture was incubated with dsRNA and [γ-^{32}P]ATP at 30°C for 20 min. For preincubation assays, peptides were incubated with PKR before addition of dsRNA and ATP. Peptide P1 consists of amino acids 77 to 113 of PKR: H-KEKKAVSPLLLTTTNSSEGLSMGNYIGLINRIAQKKR-OH. Peptide P2 consists of amino acids 230 to 254 of PKR: H-NGLRNNQRKAKRSLAPRFD LPDMKE-OH. Incubations were conducted at 30°C for 20 min unless otherwise specified, and peptides were analyzed in sodium dodecyl sulfate (SDS)–20% polyacrylamide gels, which were fixed, dried, and exposed to Kodak XAR-5 film for autoradiography. Phosphoamino acid analysis was performed as described previously (51). For assays with PKR from yeast, extracts were prepared from H1817 cultures induced with synthetic minimal medium containing 10% galactose and 2% raffinose as described previously (51) and protein concentrations were determined using a Coomassie blue dye-binding assay (Bio-Rad). Yeast extracts (50 μg of protein) were immunoprecipitated with a polyclonal anti-PKR antibody (21) and protein A-Sepharose. The immunoprecipitates were washed and then incubated as described above. To assay eIF2 phosphorylation, eIF2 was added after the initial incubation (as described in reference 33) and incubation resumed for another 20 min at 30°C.

Mutagenesis. Sites of autophosphorylation were mutated by the oligonucleotide-directed site-specific mutagenesis procedure (1, 60). Serine and threonine residues were changed to alanine to generate RBD mutant S83A/T88A/T89A/T90A, triple mutant S242A/T255A/T258A (51), and combination mutant Tri-RBD, which has all seven sites changed to alanine. Mutations were made in pUC19D (21), which contains the full-length PKR cDNA.

PKR expression in *S. cerevisiae*. PKR mutants were subcloned into pEMBLyex4 as described previously (43). Plasmids bearing the autophosphorylation site mutations were introduced into yeast strains H1816 and H1817 (13), which both have GCN2 deleted; in addition, H1817 has mutant eIF2α (S51A).

Phosphatase treatment. Total cellular protein extracts (15 μg) from transformed H1817 cells were treated with lambda phosphatase in accordance with the manufacturer's specifications (New England Biolabs) or were incubated in phosphatase buffer alone and were resolved in SDS–10% polyacrylamide gels. The proteins were then transferred to nitrocellulose for Western blotting. The membrane was incubated in 5% nonfat milk in Tris-buffered saline–Tween 20 (51) and probed with a monoclonal anti-PKR antibody (30), and the proteins were visualized by chemiluminescence (ECL; Amersham).

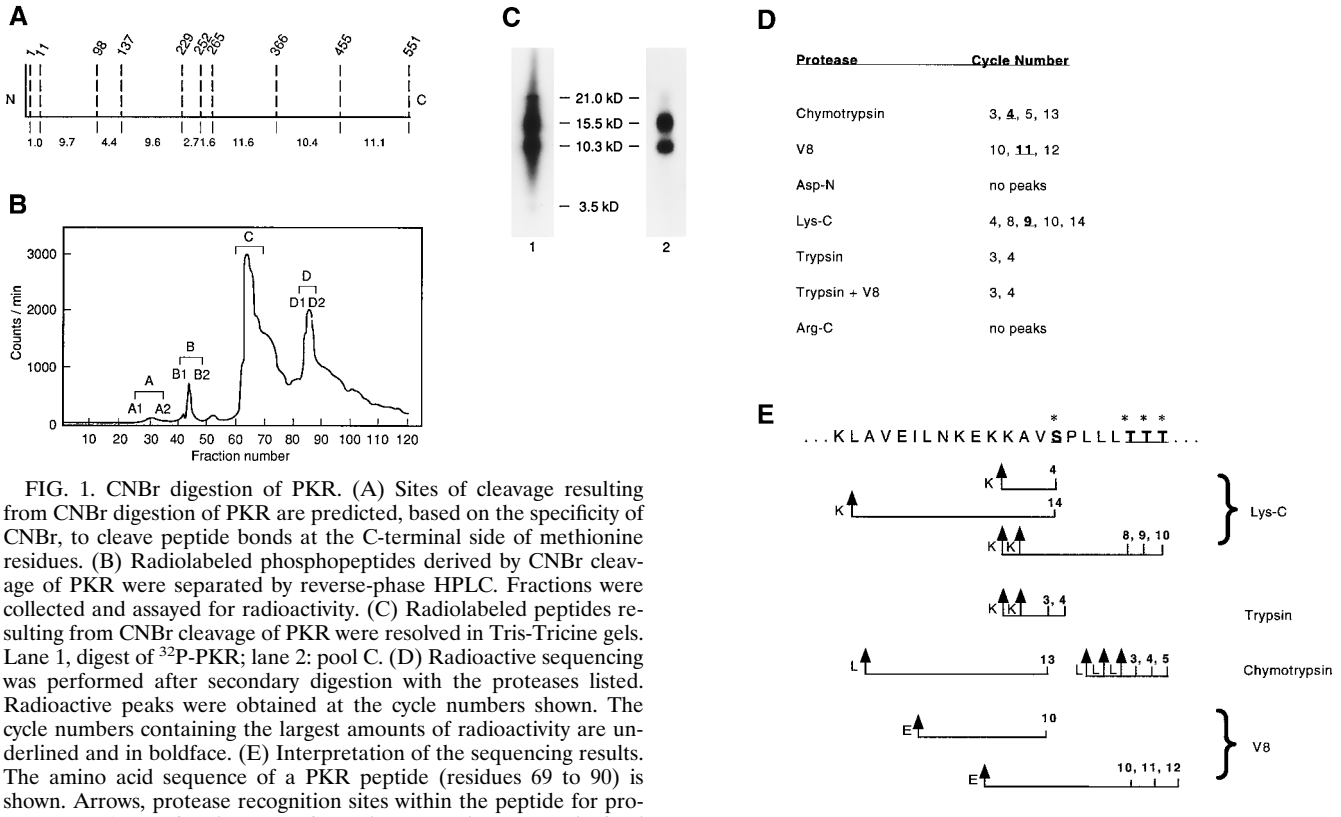


FIG. 1. CNBr digestion of PKR. (A) Sites of cleavage resulting from CNBr digestion of PKR are predicted, based on the specificity of CNBr, to cleave peptide bonds at the C-terminal side of methionine residues. (B) Radiolabeled phosphopeptides derived by CNBr cleavage of PKR were separated by reverse-phase HPLC. Fractions were collected and assayed for radioactivity. (C) Radiolabeled peptides resulting from CNBr cleavage of PKR were resolved in Tris-Tricine gels. Lane 1, digest of ^{32}P-PKR; lane 2: pool C. (D) Radioactive sequencing was performed after secondary digestion with the proteases listed. Radioactive peaks were obtained at the cycle numbers shown. The cycle numbers containing the largest amounts of radioactivity are underlined and in boldface. (E) Interpretation of the sequencing results. The amino acid sequence of a PKR peptide (residues 69 to 90) is shown. Arrows, protease recognition sites within the peptide for proteases Lys-C, trypsin, chymotrypsin, and V8. Numbers were obtained from analysis of Edman degradation reactions shown in panel D. *, autophosphorylated amino acids.

PKR expression in mammalian cells. PKR mutants were subcloned into pcDNA3 (Invitrogen). Monolayers of COS1 cells in 10-cm-diameter plates were transfected in duplicate with plasmids (15 μg) bearing the autophosphorylation site mutations, as well as a transfection efficiency control, by the calcium phosphate method (46). Cytoplasmic extracts were prepared at 48 h posttransfection, and total protein concentration was measured by a Coomassie blue assay (Bio-Rad). To assess the amount of PKR protein that was present in each sample, 100 μg of total protein was examined by Western blotting as described above. The upper half of the blot was probed with the PKR monoclonal antibody (30), and the lower half of the blot was probed with an actin polyclonal antibody (Santa Cruz Biotechnology).

HCV E2 peptide phosphorylation inhibition assay. PKR from yeast H1817 strain was autophosphorylated as in the kinase assay described above. Glutathione-coated Sepharose beads were used to purify bacterially expressed glutathione *S*-transferase (GST) protein or GST fused to the HCV E2 protein as described previously (52). The protein was eluted with glutathione, and 0.5 μg was incubated with the immunoprecipitated PKR initially for 20 min at 30°C. Poly(I:C) and [γ-^{32}P]ATP were added, and the incubation continued for another 10 min. Then peptide (3 μg) P1 or P2 was added, and incubation continued for an additional 10 min at 30°C. The reactions were analyzed as described above.

HCV E2 binding and peptide competition assay. Histidine-tagged PKR was bound to nickel-Sepharose beads as described previously (52). HCV E2 was synthesized in vitro with the T7 TNT-quick (Promega) coupled transcription-translation system in the presence of [^{35}S]methionine (New England Nuclear, DuPont). Increasing amounts of synthetic peptide P1 or P2 were added to the PKR-bound beads in 0.25 M Tris (pH 8.0)–0.1% NP-40–10 mM imidazole. Radiolabeled E2 (5 μl of the in vitro translation reaction mixture) was then added, and the mixture was incubated on ice for 2 h. The beads were washed five times in the incubation buffer (increased to contain 0.3% NP-40). The last wash was removed, and adherent labeled E2 protein was analyzed by gel electrophoresis and autoradiography.

RESULTS

Identification of PKR autophosphorylation sites. To identify the sites that are autophosphorylated when PKR is activated in vitro, we labeled PKR with [γ-^{32}P]ATP in the presence of reovirus dsRNA and then fragmented the protein with cyanogen bromide as described previously (51). The predicted pattern of PKR cleavage at methionine residues with cyanogen bromide is depicted in Fig. 1A. Nine peptides ranging from 1.0 to 11.6 kDa are expected if cleavage is complete. The products were separated by HPLC. Fractions were collected, counted by Cerenkov radiation, and pooled as shown in Fig. 1B. Four major peaks, peaks A to D, were obtained. Analysis of radioactive peaks A and B was reported previously (51). When pool C, acquired from the most prominent peak, was resolved in a Tris-Tricine gel, phosphorylated peptides migrating with apparent molecular masses of 10.3 and 15.5 kDa were observed together with a minor peptide at ~21 kDa (Fig. 1C, lane 2). Two of these peptides (15.5 and 21 kDa) probably represent partial digestion products, as the largest peptide predicted after cyanogen bromide digestion is 11.6 kDa (Fig. 1A) and secondary digestion of the 15.5-kDa peptide with cyanogen bromide yielded only a 10.3-kDa radioactive peptide (data not shown).

Phosphoamino acid analysis demonstrated that both phosphoserine and phosphothreonine were present in the 15.5- and 10.3-kDa bands (51). Pool D contained similar peptides, although the 10.3-kDa peptide was relatively less abundant (51), and pools C and D gave identical results upon subsequent analysis. A 10.3-kDa peptide was also released by cyanogen bromide treatment of phosphorylated p20, a subfragment of

PKR containing residues 1 to 184 (data not shown). As reported previously, recombinant p20 can be phosphorylated by PKR (48). This finding suggested that the 10.3-kDa peptide comes from the dsRNA-binding domain of PKR.

No peaks of radioactivity were released upon the direct sequencing of the material in pools C or D. Radioactivity did remain bound to the filter, however, indicating that the N terminus of this peptide was blocked or that the phosphoamino acids were located past the point of efficient sequencing (~25 to 30 amino acids). To test the latter possibility, material in the two pools was subjected to secondary digestion with one or two of six specific proteases. The positions of labeled amino acids were identified by counting the radioactivity of the products of sequential Edman degradation reactions. Radioactive peaks were obtained, and the results are tabulated in Fig. 1D. A summary of the sequencing data is shown in Fig. 1E. Comparison with the PKR sequence indicated that serine 83 and one or more of threonines 88, 89, and 90 were phosphorylated.

Several factors make it difficult to discriminate unambiguously among the three threonine residues, most notably the occurrence of multiple neighboring cleavage sites and the propensity of the proteases for partial cleavage. Two of the proteases used for secondary digestion recognize lysine (trypsin, Lys-C) or leucine (chymotrypsin), and incomplete digestion products were obtained because of the presence of two lysines and three leucines just upstream of the phosphorylation site (Fig. 1E). These proteases cleave at the C-terminal side of amino acids in a peptide, and in a run of identical residues they tend to cleave preferentially between the residues rather than after them. Thus the C-terminal residue in the run may not be recognized by the protease, leaving that amino acid on the N terminus and causing a shift or "stuttering" in the profiles of radioactivity obtained after Edman degradation reactions. Nevertheless, detailed examination of the profiles suggested that threonines 88, 89, and 90 are all phosphorylated. In radioactive sequencing experiments of this kind, a declining trail of radioactivity is generally observed in the Edman degradation cycles that follow the position of the radiolabeled amino acid. The pattern of increased yield followed by some carryover or "lag" derivatives (due to incomplete release of the phosphoamino acid derivative from the filter) is often used as a criterion to assess whether the yield of the phosphoamino acid is significantly greater than background. In the sequencing reactions of pools C and D with chymotrypsin, V8, and Lys-C (Fig. 1D), however, the radioactive peaks corresponding to the first threonine position were followed by two higher peaks, corresponding to the second and third threonine, and then a trail of radioactivity, indicating that all three threonines are phosphorylated. Thus we conclude that four residues, serine 83 and threonines 88, 89, and 90, located in the spacer between the two dsRBMs, are probably all phosphorylated.

Phosphorylation of PKR peptides in vitro. To corroborate the sequencing data, we tested two synthetic peptides corresponding to the RBD spacer region (P1; amino acids 77 to 113) and the central region of PKR surrounding serine 242 (P2; amino acids 230 to 254). Both P1 and P2 were phosphorylated efficiently by PKR (Fig. 2A and B), verifying that they are substrates for phosphorylation by PKR and that these sites can be recognized by intermolecular phosphorylation. Phosphoamino acid analysis (Fig. 2C) revealed that P1 is labeled on

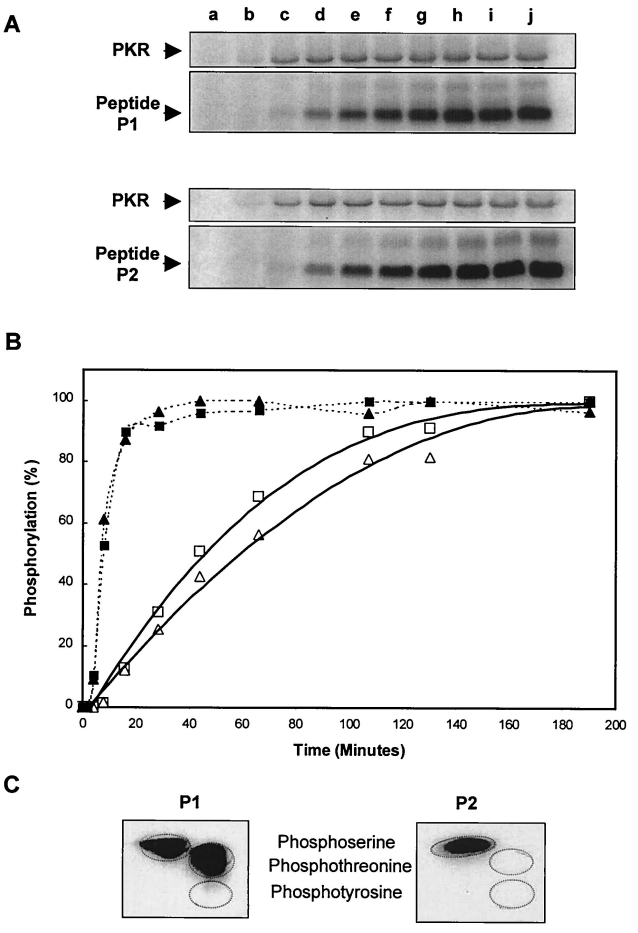
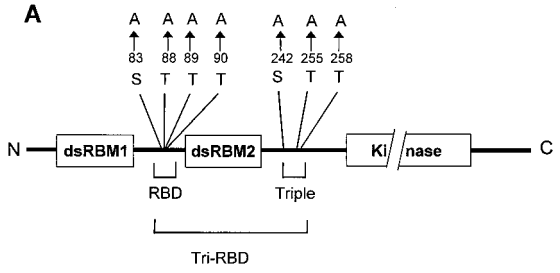


FIG. 2. Phosphorylation of peptides by PKR. (A) Kinetics of peptide phosphorylation. Reactions were stopped at different time points (a, 0 min; b, 4 min; c, 8 min; d, 16 min; e, 28 min; f, 44 min; g, 66 min; h, 108 min; I, 133 min; j, 190 min). (B) Quantitation of PKR autophosphorylation and peptide phosphorylation. Data from panel A are presented as percentages of maximal ^{32}P labeling. □, P1 phosphorylation; ■, PKR phosphorylation in the presence of P1; △, P2 phosphorylation; ▲, PKR phosphorylation in the presence of P2. (C) Phosphorylated peptides were subjected to phosphoamino acid analysis by hydrolysis, two-dimensional separation, and autoradiography. Positions of markers, detected by ninhydrin staining, are circled.

both the serine and the threonine residues whereas P2 is labeled on serine only, as expected. The phosphorylation of P1 and P2 continued after PKR autophosphorylation reached saturation (Fig. 2B); under these conditions, neither of the peptides interfered with PKR autophosphorylation (data not shown), suggesting that they do not compete effectively with the activation reaction.

Autophosphorylation sites in the RNA-binding domain do not affect PKR function in yeast. To examine the significance of the four autophosphorylation sites in the RBD spacer, the serine and three threonine residues were all changed to alanine (Fig. 3A). This PKR RBD mutant (S83A/T88A/T89A/T90A) was tested in yeast together with wild-type PKR and three other mutants: K296R, which is inactive as a result of a mutation in the ATP-binding/phosphotransfer site (23); Triple mutant S242A/T255A/T258A, which is partially inactivated by three mutations in the central region (51); and Tri-RBD, which



B **Yeast Growth Assay**

	SD	SGAL	SD + 3AT
WT	+	−	+
K296R	+	+	−
RBD	+	−	+
Triple	+	−	±
Tri-RBD	+	−	±

FIG. 3. PKR mutations and yeast growth. (A) Schematic of PKR autophosphorylation site mutations. Serine and threonine residues were changed to alanine to generate Triple and RBD mutants and the combination mutant; Tri-RBD, which has all seven sites changed to alanine. (B) Results of yeast growth assays. Transformed cells (H1816) were grown on SD medium and were replica plated to SGAL and SD+3AT medium for growth analysis (43). +, strong growth; ±, slow growth; −, no growth.

contains all seven of the mutations in the Triple and RBD mutants.

Transformants of yeast strain H1816, which carries a wild-type eIF2α allele, were grown on selective media to analyze mutant kinases for their activity (13). Yeast cells that express fully active PKR cannot grow on galactose medium (SGAL) due to overexpression of PKR (driven by the Gal promoter) and consequent phosphorylation of eIF2α. In contrast, expression of PKR at much lower levels on dextrose-containing medium allows cells to grow in the presence of 3-aminotriazole (SD+3AT), an inhibitor of histidine biosynthesis, because a moderate level of eIF2 phosphorylation allows for GCN4 translation (Fig. 3B). Cells transformed with inactive PKR mutants, such as K296R, grow on both synthetic minimal dextrose (SD) and SGAL media but not on SD+3AT (Fig. 3B). The phenotype of yeast cells transformed with the RBD mutant was indistinguishable from that of cells transformed with wild-type PKR, suggesting that the RBD mutant is fully active. Furthermore, the slow-growth phenotype conferred by the Triple mutant in SD+3AT medium (51) was not exacerbated in the Tri-RBD mutant (Fig. 3B). This slow-growth phenotype is attributable to mutations in the central region, most notably T258A, which is important to the activation of PKR (51). These data indicate that phosphorylation of residues in the RBD spacer is not required for PKR function in yeast cells.

Phosphorylation state of PKR in vivo. To determine specifically whether these residues are required for autophosphorylation of PKR in vivo, yeast strain H1817 was transformed with wild-type or mutant forms of PKR. This strain contains the S51A mutation of eIF2α, allowing for high expression of both active and inactive PKR (13). The yeast was grown under inducing conditions (43), and extracts were treated with lambda phosphatase or were incubated with phosphatase buffer alone. These extracts were then resolved in an SDS gel, transferred to nitrocellulose, and probed with monoclonal anti-PKR antibody.

Wild-type PKR showed a distinct increase in gel mobility upon phosphatase treatment (Fig. 4A), as observed previously (42). Catalytically inactive mutant K296R migrated with the phosphatase-treated form and showed no shift in mobility upon phosphatase treatment, indicating that yeast does not contain endogenous kinases that phosphorylate PKR. When the Tri-RBD mutant, carrying seven alanine substitutions, was treated with phosphatase, it showed a small but reproducible shift in mobility (Fig. 4A), indicating that the mutant kinase is phosphorylated to some extent in vivo. This conclusion was confirmed by labeling with ^{32}P in vivo and analysis of peptides derived by cyanogen bromide cleavage. Consistent with the removal of the seven sites of phosphorylation in the RBD and central region, phosphopeptides of 3.5, 10.3, and 15.5 kDa were missing. The digest did, however, yield the 21-kDa peptide and a peptide migrating faster than 10 kDa (data not shown), presumably attributable to sites in the activation loop of the kinase domain (42) or to other potential phosphorylation sites reported previously (58).

PKR kinase activity in vitro. Although these assays suggested that the RBD mutation did not affect kinase activity or autophosphorylation in yeast, we wanted to measure the activities of the mutant kinases directly. PKR was isolated by immunoprecipitation from extracts of H1817 strains carrying mutant or wild-type forms of the enzyme. The amount of PKR protein recovered was normalized by immunoblotting (as shown in Fig. 4A), and its activity was assessed in kinase assays. Figure 4B shows that wild-type PKR was active for autophosphorylation and for phosphorylation of eIF2α, while K296R was inactive in both respects. The Triple mutant was weakly active, and S242A was highly active, as expected from published results (51). Consistent with the in vivo data, the RBD mutations alone did not affect PKR autophosphorylation or the phosphorylation of eIF2α and modestly exacerbated the

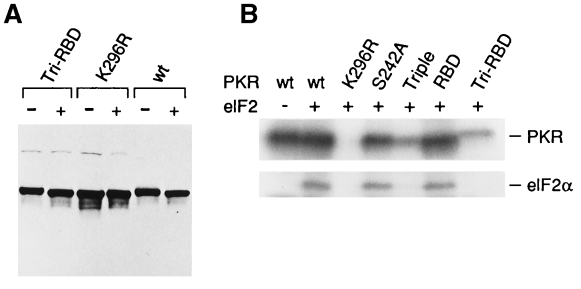


FIG. 4. Properties of PKR mutants. (A) PKR phosphorylation state. Total cellular protein extracts from transformed H1817 cells were treated with lambda phosphatase (+) or phosphatase buffer alone (−), resolved in SDS–10% polyacrylamide gels, and analyzed by Western blotting with a monoclonal anti-PKR antibody (30) and chemiluminescence detection. wt, wild type. (B) Kinase activity. Autophosphorylation of wild-type and mutant PKR and phosphorylation of eIF2α were conducted in vitro. Kinase assay mixtures contained PKR, isolated on antibody-coated beads, in the presence (+) or absence (−) of eIF2. Detection was by gel electrophoresis and autoradiography.

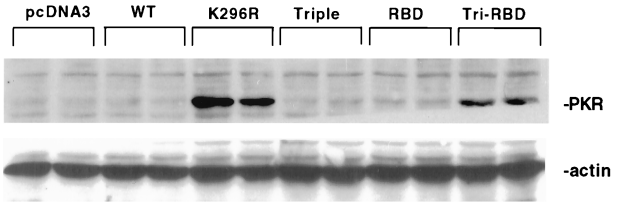


FIG. 5. Expression of PKR mutants in mammalian cells. COS1 cells were transfected in duplicate with plasmids encoding wild-type (WT) or mutant PKR together with pEGFP-C1 (Clontech) encoding green fluorescent protein. Transfection efficiency was monitored by observing the ratio of green fluorescent cells to the total number of cells. Protein expression was monitored by immunoblotting with a PKR monoclonal antibody (30) and an antiactin antibody.

effects of the Triple mutation when the mutations were present together in Tri-RBD (Fig. 4B). Thus, the RBD sites influence kinase activity modestly in vitro.

In view of their location, we also considered the possibility that the RBD phosphorylation sites might play a role in RNA binding. To address this possibility, we measured the ability of immobilized PKR, isolated by immunoprecipitation from yeast as described above, to interact with ^{32}P-labeled synthetic dsRNA. Mutations in the RBD did not significantly alter the retention of dsRNA by PKR (data not shown).

Expression of PKR mutants in mammalian cells. To examine the effect of the RBD mutations in mammalian cells, we measured the levels of mutant PKR during transient expression assays (Fig. 5). Cytoplasmic extracts were prepared from transfected COS1 cells, and equal amounts of total proteins were resolved in a gel; PKR expression was monitored by Western blotting. Plates were transfected in duplicate, and the immunoblot was probed with antibodies against human PKR (top) and actin (bottom). As observed previously (42, 54) PKR mutant K296R was expressed at a much higher level than wild-type PKR, indicating that this mutant is unable to downregulate its expression because it is inactive. Both the Triple and RBD mutants were expressed at very low levels, indicating that these mutants are active. The Tri-RBD mutant, however, was expressed at an intermediate level, lower than that for K296R but higher than those for the other mutants, indicating that this mutant is less active than either the Triple or RBD mutant but not as inactive as K296R. Thus, the RBD sites contribute to, but are not essential for, the activity of the kinase.

Peptide competition and HCV E2 inhibition. We previously showed that HCV E2 binds to PKR and inhibits its kinase activity, as measured by its ability both to become autophosphorylated and to phosphorylate a histone H2a substrate (52). To determine whether HCV E2 can inhibit phosphorylation of peptides corresponding to its autophosphorylation sites, activated PKR was incubated with E2 in a kinase assay containing P1 or P2. Figure 6A shows that GST-E2 efficiently blocked the phosphorylation of peptide P1 (lane 6). The phosphorylation of P2 was also inhibited but to a lesser extent (lane 7), but GST protein did not significantly inhibit P1 or P2 phosphorylation (lanes 3 and 4). As discussed previously (52), GST-E2 did not affect PKR autophosphorylation in this system due to its preactivation state.

Deletion of or mutations in the PePHD in HCV E2 abrogate its functional interaction with PKR (52). We therefore tested the ability of the two PKR peptides to interfere with the binding of E2 to PKR. As shown in Fig. 6B, the interaction between PKR and HCV E2 was not blocked by either peptide P1 or P2. Concentrations of P1 as high as 60 mg/ml failed to prevent the binding of E2 to PKR. Thus E2 is a strong ligand for PKR and can compete with substrates that are *trans*-autophosphorylation sites, but it is not displaced from PKR by high concentrations of peptide substrates corresponding to these sites.

DISCUSSION

PKR is found in most mammalian cells at a low level, and expression of the enzyme is induced by interferon. The kinase is activated by viral infection in cells and by binding to dsRNA in vitro (33). Autophosphorylation on several serine and threonine residues, which accompanies activation (18, 29), is believed to be instrumental in converting the enzyme to a state in which it is able to phosphorylate its substrates including eIF2. Previously we identified three autophosphorylation sites in the central region of PKR and one or two sites in the kinase

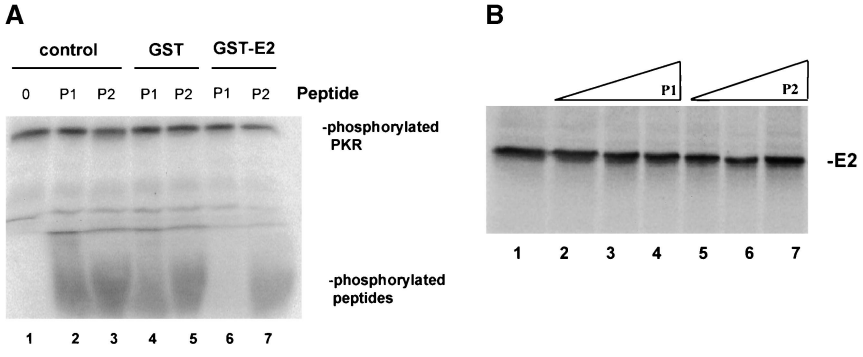


FIG. 6. HCV E2 competition with PKR autophosphorylation site peptides. (A) Kinase assay mixtures contained PKR immunoprecipitated from yeast extracts. PKR was incubated with GST or GST-E2 proteins and peptide P1 or P2 before adding [^{32}P]ATP. Incorporation of ^{32}P into PKR and peptides was monitored by gel electrophoresis and autoradiography. (B) E2 binding assays. Nickel nitrilotriacetic acid-agarose-bound histidine-tagged PKR was incubated with peptide P1 (1, 5, 15, and 60 mg/ml [lanes 2 to 5]) or peptide P2 (0.6 and 2.4 mg/ml [lanes 6 and 7]) or without added peptide (lane 1) and then with ^{35}S-labeled E2 protein. Binding was monitored by gel electrophoresis and autoradiography.

TABLE 1. PKR phosphorylation sites[a]

Protein and amino acid	Sequence
eIF2α	L S E L **S** R R R I
Tat	G Q **S** S **T** H Q V **S**
p53	E G P D **S** D
PKR	
Ser 242	K A K R **S** L A P R
Thr 255	D M K E **T** K Y T V
Thr 258	E T K Y **T** V D K R
Ser 83	K K A V **S** P L L L
Thr 88, 89, 90	P L L L **T** **T** **T** N S
Thr 446	D G K R **T** R S K G
Thr 451	R S K G **T** L R Y M

[a] Sequences surrounding known PKR phosphorylation sites within PKR itself and in its substrates eIF2α, human immunodeficiency virus type 1 Tat, and p53 are aligned. Phosphorylated amino acids are in boldface. Uncharged (L and V) and basic (K or R) amino acids within 1 to 3 residues of phosphorylation sites are underlined.

domain that participate in the activation of the enzyme (42, 51). These sites did not appear to be the most prominent autophosphorylation sites in vitro, however (51). Data presented here identify amino acids in the spacer region of the RBD, serine 83 and threonines 88, 89, and 90, as major sites of phosphorylation in vitro. Mass spectrometry analysis of PKR isolated from yeast demonstrated that phosphorylation of the same sites also occurs in vivo (58).

The mutagenic analysis reported here suggests that the autophosphorylation sites in the RBD are involved in, but are not required for, kinase activation. This inference is supported by the observation (31) that large deletions in the RBD did not affect activation of the kinase when endogenous PKR was present. In these experiments the activation of mutant PKR that could not become activated through dsRNA binding (in the absence of the RBD) was presumably brought about either through *trans*-phosphorylation by endogenous PKR or by removal of an autoinhibitory domain located in the RBD. Evidence in support of the latter mechanism comes from the finding that deletions within the RBD can lead to elevated kinase activity in vitro (57, 59). These data imply the existence of a negative regulatory element in the RBD; by extension, dsRNA binding may serve as a trigger to relieve the inhibition (41).

In combination with sites in the central region of PKR, the RBD autophosphorylation sites appear to play a role in activation of the kinase in vitro and in mammalian cells. They do not affect kinase activity to a detectable extent in the yeast growth assay, however, where subtle changes in kinase activity or RNA-binding affinity may be missed because of the abundance of dsRNA molecules in yeast. It is possible that the small decrease in activity reflects a change in dsRNA binding or release due to the additional negative charge provided by the addition of phosphate groups to the RBD sites. Efficient activation of PKR by dsRNA requires two dsRBMs and is adversely affected by a large deletion in the spacer between them, although a small deletion was tolerated (21). A recent structural study of the RBD by nuclear magnetic resonance techniques indicates that the spacer constitutes a flexible linker between the dsRBMs, allowing the RBD to wrap around the dsRNA (35). Thus it is conceivable that phosphorylation in the spacer limits this flexibility. Such constraints might have several consequences for the specificity of RNA binding. First, the constraints could restrict the size range of dsRNA molecules that can interact with PKR; second, in view of the growing body of evidence that PKR can be activated or blocked by molecules that are not fully duplexed (3, 9), the constraints might impose restrictions on the spectrum of partially duplexed RNAs that serve these functions; third, they might aid in discriminating between activators and inhibitors. Furthermore, it is possible that PKR may need to dissociate from structures with which it interacts in vivo to *trans*-phosphorylate substrates or other PKR molecules. Since RBD phosphorylation does not reduce PKR's RNA-binding affinity significantly, there is no evidence that it participates in the release of PKR from dsRNA, but phosphorylation of PKR may influence its functional interaction with ribosomes or its distribution or transport between subcellular compartments.

We note that PKR is activated by autophosphorylation of the third basic domain and the activation loop in the kinase domain (42, 51). Our finding that PKR can *trans*-phosphorylate peptides that contain PKR autophosphorylation sites, even after PKR autophosphorylation has reached saturation, indicates that these sites (serine 242, serine 83, and threonines 88, 89, and 90) can be phosphorylated through an intermolecular mechanism.

Together with the four to five sites identified previously (42, 51) the present work brings the total of PKR autophosphorylation sites identified to date to nine. Within this set, threonines outnumber serines by seven to two. Previous reports have demonstrated that PKR yields considerably more phosphoserine than phosphothreonine (29), so it seems likely that additional sites remain to be characterized. The sequences surrounding the known autophosphorylation sites are listed and aligned in Table 1. Among the PKR substrates, phosphorylation sites have been identified for the α subunit of eIF2 (39), for Tat (5), and for p53 (11); these sites are also included in Table 1.

Little homology is readily apparent among the sites, but, strikingly, eight amino acids in the phosphorylated region of the RBD are identical to residues in the HCV envelope protein, E2 (Table 2). Moreover, the homology extends upstream. These observations led to the discovery that the E2 protein from IFN-resistant strains of HCV is an inhibitor of and potential substrate for PKR (52). Previously it was shown that HCV E2 inhibits PKR autophosphorylation and substrate phosphorylation in vitro (52). Here we show that HCV E2 binds efficiently to PKR and that this interaction cannot be dissociated by a peptide (P1) that contains the homologous PKR sequence, even though P1 contains autophosphorylation sites and is a *trans*-phosphorylation substrate. Nevertheless, phosphorylation of this peptide is efficiently inhibited by E2,

TABLE 2. HCV E2 sequence alignment[a]

Protein	Sequence
PKR	E I L N K E K K A V S P L L L T T T
	* * * * * * * ! ! ! ! ! ! !
E2	D L E D R D R S E L S P L L L T T T

[a] Alignment between amino acids 73 to 90 of PKR, including the RBD phosphorylation sites (underlined) and amino acids 270 to 287 of the HCV E2 protein. *, conserved amino acids; !, identical amino acids.

indicating that E2 is a potent inhibitor of PKR autophosphorylation as well as substrate phosphorylation and that this action is not carried out through a simple competitive mechanism. These data imply that mimicry of the autophosphorylation site by HCV E2 contributes to its inhibition of PKR via a pseudosubstrate mechanism. In addition, other regions of the E2 protein may also be involved, possibly acting to stabilize the PKR-E2 interaction by reducing the "off" rate. Thus, duplication of a major PKR autophosphorylation site is part of the strategy evolved by HCV for evading the effects of IFN, but further work is required to fully uncover this aspect of the E2 protein's function.

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