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Mechanism of Interferon Action: Evidence for Intermolecular Autophosphorylation and Autoactivation of the Interferon-Induced, RNA-Dependent Protein Kinase PKR

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The interferon-induced RNA-dependent protein kinase (PKR) is postulated to have an important regulatory role in the synthesis of viral and cellular proteins. Activation of the enzyme requires the presence of a suitable activator RNA and is accompanied by an autophosphorylation of PKR. Active PKR phosphorylates the α subunit of protein synthesis eukaryotic initiation factor 2, resulting in an inhibition of translation initiation. The mechanism of autophosphorylation is not well understood. Here we present evidence that the autophosphorylation of human PKR can involve intermolecular phosphorylation events, i.e., one PKR protein molecule phosphorylating a second PKR molecule. Both wild-type PKR and the point mutant PKR(K296R) synthesized in vitro were phosphorylated, even though PKR(K296R) was deficient in kinase catalytic activity. Phosphorylation of both wild-type PKR and PKR(K296R) was inhibited in the presence of 2-aminopurine. Furthermore, purified human recombinant PKR(K296R) was a substrate for the purified wild-type human PKR kinase. This intermolecular phosphorylation of mutant PKR(K296R) by wild-type PKR was dependent on double-stranded RNA and was inhibited by 2-aminopurine. Finally, PKR mRNA was capable of mediating an autoactivation of wild-type PKR kinase autophosphorylation in vitro.

The RNA-dependent protein kinase PKR (also termed P1/eIF-2α protein kinase, p68 kinase, DAI, and dsI) has a central role in regulation of protein synthesis in virus-infected cells (16, 21, 22, 23, 26). Treatment of cells with type I interferon (IFN) results in an increased level of PKR protein, mainly due to induction of PKR gene transcription (19, 28). The newly synthesized PKR protein is catalytically inactive; it requires RNA to be converted to the catalytically active form. Activation of PKR enzymic function presumably results from an RNA-mediated conformational change of the protein affecting the kinase catalytic subdomains. This is implied by the result that ATP can be cross-linked to the kinase only in the presence of double-stranded RNA (dsRNA) (3, 8), suggesting that RNA-mediated allosteric changes affect PKR conformation in regions including the ATP-binding site. Furthermore, gel retardation experiments indicate that activation of PKR with dsRNA of a chain length greater than 67 to 85 bp results in the appearance of protein-RNA complexes with different electrophoretic mobilities, consistent with the notion that conformational changes occurred (18). Activation of PKR enzymic activity coincides with autophosphorylation of the PKR protein at serine and threonine residues (8, 15). Activated PKR catalyzes the phosphorylation of protein synthesis eukaryotic initiation factor 2 (eIF-2) at serine 51 of the α subunit. This modification of eIF-2 leads to the formation of a stable complex between eIF-2 and the guanine nucleotide exchange factor eIF-2B, which results in the inhibition of mRNA translation (5, 11). In addition to regulation of translation in virus-infected cells, PKR has also been implicated in other cellular activities, such as cell differentiation (17) and proliferation (13, 20).

The mechanism of PKR autophosphorylation and subsequent kinase activation is not well understood. Herein we provide insights regarding the autophosphorylation mechanism. We demonstrate that both the wild-type (wt) PKR kinase and the catalytic activity-deficient mutant PKR(K296R) are phosphorylated when synthesized in the rabbit reticulocyte lysate cell-free system. And, more importantly, highly purified wt PKR catalyzes the phosphorylation of highly purified, tagged recombinant PKR(K296R), thereby definitively establishing that PKR undergoes intermolecular autophosphorylation events. Furthermore, we provide evidence that PKR mRNA is an efficient activator RNA of the PKR autocatalytic kinase activity.

Wt recombinant PKR synthesized in vitro is phosphorylated. As an extension of our expression studies of the human IFN-induced PKR (28, 29), we examined the phosphorylation state of in vitro-synthesized PKR. Previous work has shown that the enzymically inactive (unphosphorylated) form of PKR can be distinguished from the enzymically active (phosphorylated) form by virtue of the different mobilities of the two forms in sodium dodecyl sulfate-polyacrylamide gel electrophoretic (SDS-PAGE) gels (8, 15). We utilized this difference in electrophoretic mobility to assess the apparent phosphorylation state of human PKR synthesized in vitro in the rabbit reticulocyte lysate system in the presence of agonists and antagonists of the kinase. ^{35}S-labeled proteins synthesized by reticulocyte lysates programmed with in vitro-transcribed PKR RNA were analyzed by SDS-PAGE (7.5% polyacrylamide gel) followed by fluorography (Fig. 1). Wt PKR synthesized in the absence of added effectors displayed a retarded mobility (Fig. 1, lane 4) relative to wt PKR that had been treated with calf intestinal phosphatase (CIP) subsequent to the synthesis (lane 5). Thus, it appears that in vitro-synthesized wt PKR is phosphorylated and that treatment with CIP dephosphorylated the wt protein, resulting in an increased electrophoretic mobility. The presence of 2-aminopurine (2AP) during the in vitro translation resulted in the synthesis of a wt PKR product that likewise displayed an increased mobility (Fig. 1, lane 6),

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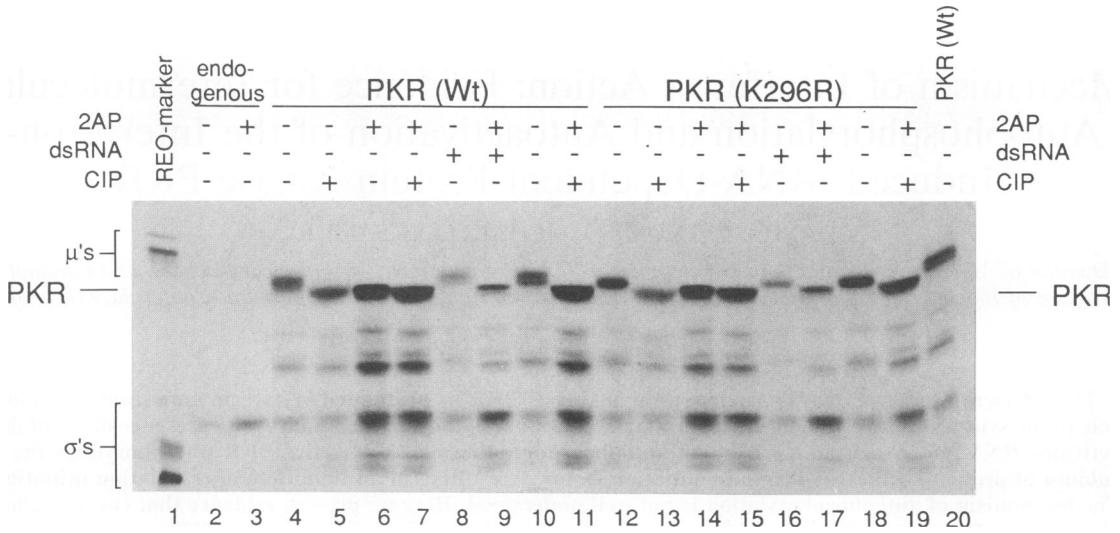

FIG. 1. In vitro-synthesized wt PKR [PKR(Wt)] and catalytic activity-deficient mutant PKR(K296R) are phosphorylated proteins. The plasmids pBlue-P1KIN (28) and pBlue-P1KIN(K296R) (29), containing the entire reading frames of the human wt PKR and the phosphotransfer mutant PKR(K296R), respectively, served as templates for in vitro transcription of PKR RNAs with T7 RNA polymerase as previously described (28). The PKR RNAs were utilized to program the in vitro synthesis of PKR protein in micrococcal nuclease-treated rabbit reticulocyte lysate (Promega). Translation reaction mixtures contained either no exogenously added RNA (endogenous; lanes 2 and 3), in vitro-transcribed wt PKR RNA (lanes 4 to 11 and 20), or PKR(K296R) RNA (lanes 12 to 19) at 4 μg/ml. Reaction mixtures were incubated for 1 h at 30°C with no further additions or with 10 mM 2AP (lanes 6, 7, 9, 11, 14, 15, 17, and 19) or 0.5 μg of poly(rI) · poly(rC) per ml (dsRNA; lanes 8, 9, 16, and 17) as indicated. After incubation, some reaction mixtures were further incubated with 20 U of CIP (lanes 5, 7, 11, 13, 15, and 19) for 1 h at 37°C. Products were analyzed on an SDS-PAGE (7.5% polyacrylamide) gel, and ^{35}S-labeled proteins were visualized by fluorography. Lane 1 contained a reovirus (REO) protein standard, and the positions of the σ- and μ-class proteins are indicated. The position of full-length PKR, as determined by Western analysis (28), is also indicated.

possibly because 2AP inhibited the autophosphorylation of the newly synthesized PKR. Furthermore, the amount of PKR synthesized in vitro was increased by the addition of 2AP to the reaction mixture (Fig. 1, lane 6). Treatment with CIP of wt PKR synthesized in the presence of 2AP did not further affect the mobility of the protein (Fig. 1, lane 7), indicating that 2AP substantially inhibited the phosphorylation of PKR.

It should be noted that phosphorylation of wt PKR in the translation reaction mixture occurred in the absence of any exogenously added dsRNA. Furthermore, addition of poly(rI) · poly(rC) dsRNA yielded PKR protein (Fig. 1, lane 8) that had a mobility comparable to that of the PKR synthesized in the absence of poly(rI) · poly(rC) (lanes 4 and 10), although the amount of wt PKR product was reduced in reaction mixtures to which dsRNA was added. Addition of dsRNA did not prevent inhibition of phosphorylation by 2AP (Fig. 1, lane 9), suggesting a dominant effect of 2AP. Because the addition of poly (rI) · poly(rC) did not further promote PKR phosphorylation but 2AP blocked PKR phosphorylation, some RNA capable of activating PKR was likely already present in the reaction mixture.

The catalytic activity-deficient mutant PKR(K296R) synthesized in vitro is phosphorylated. Arginine substitution of the conserved lysine in PK domain II results in an inactivation of most kinases (10), including PKR (12). We therefore examined the phosphorylation state of the mutant PKR(K296R) (29) synthesized in vitro (Fig. 1). Interestingly, mutant PKR(K296R) migrated with a reduced mobility in the SDS-PAGE gel (Fig. 1, lanes 12 and 18) relative to the K296R protein treated with CIP (lane 13). However, the mobility of the mutant PKR (K296R) protein (Fig. 1, lanes 12 and 18) was slightly faster than that of wt PKR synthesized in the absence of any effectors (lanes 4, 10, and 20), suggesting that PKR(K296R) may be phosphorylated less extensively than wt PKR. Phosphorylation of mutant PKR(K296R) was completely inhibited by 2AP (Fig. 1, lanes 14 and 15), mimicking the result obtained with wt PKR (lanes 6 and 7). Addition of poly(rI) · poly(rC) to the in vitro translation reaction mixture did not increase the phosphorylation of mutant PKR(K296R) (Fig. 1, lane 16), and 2AP was still able to inhibit phosphorylation of PKR(K296R), even in the presence of poly(rI) · poly(rC) (lane 17).

The results obtained from our study of the in vitro synthesis of mutant PKR(K296R) closely resembled those obtained with wt PKR and may be summarized as follows. (i) In the absence of any agonists or antagonists of PKR, both the wt and K296R mutant proteins were phosphorylated, but the mutant PKR(K296R) appeared to be phosphorylated to a lesser degree than the wt PKR. (ii) Phosphorylation of both wt and K296R mutant PKR proteins was inhibited when 2AP, an antagonist of PKR activity, was included in the translation reaction mixture. (iii) Addition of activator dsRNA to the translation mixture did not result in an increased phosphorylation of either wt PKR or mutant PKR(K296R). These observations raise two important questions. What is the source of the enzyme activity that was responsible for phosphorylation of the catalytically inactive PKR(K296R) protein? What is the nature of the endogenous RNA that activated wt PKR in the in vitro translation system in the absence of exogenously added dsRNA?

Our observation that in vitro-synthesized mutant PKR (K296R) was phosphorylated (Fig. 1) appears to conflict with the observation of Katze et al. (12), who reported that PKR(K296R) possesses no residual kinase catalytic activity. This apparent inconsistency can be resolved if some kinase endogenous to the rabbit reticulocyte system was responsible for catalyzing the phosphorylation of PKR(K296R). A clue to

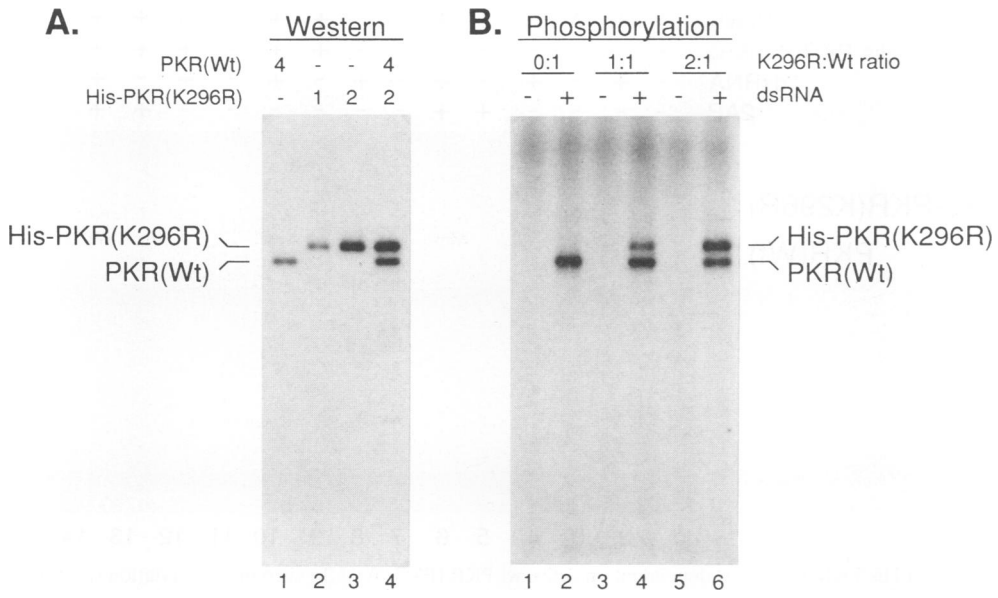

FIG. 2. His-PKR(K296R) is a substrate for phosphorylation in vitro by wt PKR [PKR(Wt)] kinase. (A) Western immunoblot analysis of PKR(Wt) and His-PKR(K296R). Proteins were fractionated on an SDS-PAGE (10% polyacrylamide) gel and electroblotted to nitrocellulose, and then PKR proteins were detected with polyclonal anti-PKR rabbit antiserum (28) and ^{125}I-labeled protein A. Lane 1 contained 4 μl of purified PKR(Wt), lanes 2 and 3 contained 1 and 2 μl, respectively, of purified His-PKR(K296R), and lane 4 contained 4 μl of PKR(Wt) and 2 μl of His-PKR(K296R). (B) In vitro phosphorylation of PKR(Wt) and His-PKR(K296R). Fifteen-microliter in vitro phosphorylation reaction mixtures (25) contained 0.5 μl of purified PKR(Wt) and the following additions: no activator RNA (lanes 1, 3, and 5); 0.1 μg of poly(rI) · poly(rC) per ml (dsRNA; lanes 2, 4, and 6); and His-PKR(K296R) (lanes 3 to 6) at equimolar or double-molar amounts (relative to wt PKR) as indicated. Lanes 1 and 2 contained only PKR(Wt). Reaction mixtures were incubated for 2 min at 30°C. Samples were fractionated on an SDS-PAGE (10% polyacrylamide) gel, and ^{32}P-labeled phosphoproteins were visualized by autoradiography. The positions of the 67-kDa PKR(Wt) and the 71-kDa His-PKR(K296R) protein bands are indicated. PKR(Wt) kinase, generously provided by S. McCormack of this laboratory, was purified chromatographically from ribosomal salt washes of IFN-treated human amnion U cells as previously described for PKR from mouse fibroblasts (2), except that the procedure was modified to include a MonoQ ion-exchange fast protein liquid chromatography (FPLC) fractionation step. The cDNA encoding PKR(K296R) was cloned in the pBlueBacHisC vector (Invitrogen), and a recombinant baculovirus was produced. Sf21 insect cells were infected at a multiplicity of infection of >10, and His-PKR(K296R) protein was purified from the 100,000 × *g* supernatant by Ni^{2+}-chelating Sepharose column chromatography, followed by MonoS ion-exchange FPLC. The purifications of both PKR(Wt) and His-PKR(K296R) will be described in greater detail elsewhere.

the nature of this endogenous rabbit kinase comes from the result that phosphorylation of PKR(K296R) was inhibited by 2AP, an inhibitor that is relatively specific for PKR (6, 30). Conceivably the endogenous rabbit PKR was activated by the exogenously added human PKR mRNA and subsequently catalyzed the phosphorylation of the synthesized human PKR(K296R) protein. Because Katze and coworkers immunoprecipitated the in vitro-synthesized human PKR(K296R) from the reticulocyte reaction mixture prior to the phosphorylation reaction by using a monoclonal antibody selective for the human PKR protein (12), the endogenous rabbit PKR was likely absent from their phosphorylation reaction mixtures and thus no *trans*-phosphorylation of PKR(K296R) was observed by them (12).

Human His-PKR(K296R) is phosphorylated in vitro by wt PKR. To directly address the question of whether the mutant PKR(K296R) is a substrate of wt PKR, we carried out in vitro phosphorylation experiments with highly purified human PKR proteins. Mixing experiments were performed with wt PKR and mutant PKR(K296R). Because wt PKR and the mutant PKR(K296R) are immunologically and electrophoretically indistinguishable (29), we engineered a PKR(K296R) fusion protein with a histidine tag that both altered electrophoretic mobility and facilitated purification. The cDNA encoding the human PKR(K296R) was cloned into the pBlueBacHisC vector (Invitrogen), and a recombinant baculovirus, Ac*M*NPV [νHis-PKR(K296R)], was produced. Insect Sf21 cells infected with this recombinant virus synthesized PKR(K296R) fused to an N-terminal tag containing a polyhistidine sequence and an enterokinase cleavage site. More importantly, this tag added about 4 kDa to the size of PKR and thus permitted a clear distinction on SDS-PAGE gels between the mutant His-PKR(K296R) and the wt PKR. This is demonstrated by the Western immunoblot shown in Fig. 2A. Wt PKR (Fig. 2A, lanes 1 and 4) and His-PKR(K296R) (lanes 2 to 4) were detected as two discrete protein bands of about 67 and 71 kDa, respectively.

In vitro phosphorylations with [γ-^{32}P]ATP were then carried out as indicated, either in the absence or the presence of activator dsRNA (Fig. 2B). Purified wt PKR autophosphorylated in an RNA-dependent manner (Fig. 2B, lanes 1 and 2) as expected. When the His-PKR(K296R) protein was added to the reaction mixture in an equimolar amount (Fig. 2B, lanes 3 and 4) or at twice the molar amount of wt PKR (lanes 5 and 6), the appearance of a phosphoprotein that was slightly larger than wt PKR was observed in reaction mixtures containing dsRNA (lanes 4 and 6). Reaction mixtures lacking poly(rI) · poly(rC) dsRNA (Fig. 2B, lanes 3 and 5) did not contain phosphorylated product, either wt PKR or His-PKR(K296R). The wt PKR and His-PKR(K296R) phosphoproteins (Fig. 2B, lane 4) aligned with the Western signals of wt PKR and His-PKR(K296R) (Fig. 2A, lane 4), respectively.

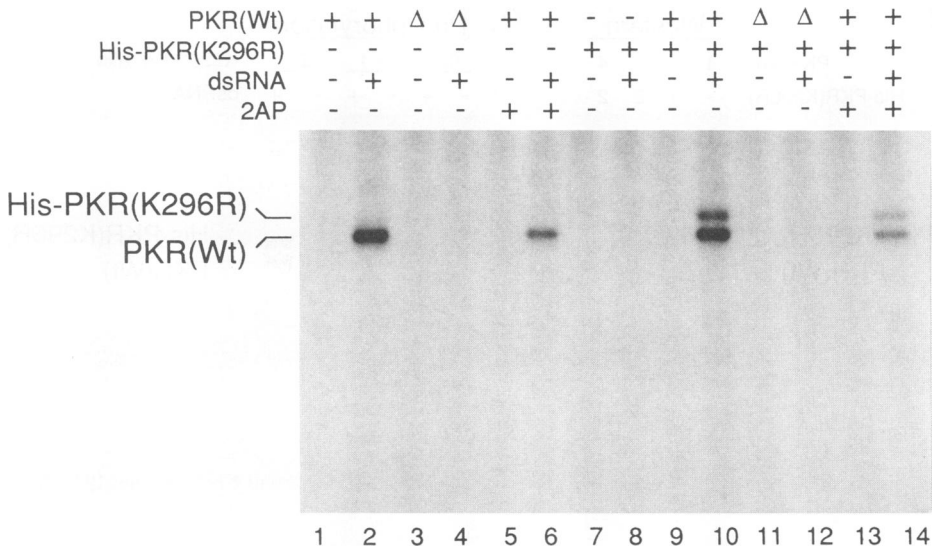

FIG. 3. Phosphorylation of His-PKR(K296R) is dependent on active wt PKR [PKR(Wt)]. In vitro phosphorylation reaction mixtures contained 0.1 μg of poly(rI) · poly(rC) per ml as indicated (dsRNA). Lanes 1 to 6 contained purified PKR(Wt); for lanes 3 and 4, as indicated by the triangle, PKR(Wt) was incubated at 65°C for 5 min prior to the in vitro phosphorylation reaction. Lanes 5 and 6 contained 10 mM 2AP, lanes 7 and 8 contained a molar amount of purified His-PKR(K296R) similar to the amount of PKR(Wt) in lanes 1 to 6, and lanes 9 to 14 contained equimolar amounts of PKR(Wt) and His-PKR(K296R). For lanes 11 and 12, PKR(Wt) was heat inactivated, and lanes 13 and 14 contained 10 mM 2AP. ^{32}P-labeled phosphoproteins were fractionated on an SDS-PAGE (10% polyacrylamide) gel and visualized by autoradiography. The positions of the 67-kDa PKR(Wt) and the 71-kDa His-PKR(K296R) protein bands are indicated.

Furthermore, both phosphoproteins were immunoprecipitated with PKR-specific antiserum (data not shown). These results suggest that His-PKR(K296R) is a substrate of the wt PKR kinase.

Phosphorylation of His-PKR(K296R) is dependent upon active wt PKR. The phosphorylation of His-PKR(K296R) was dependent upon the presence of active wt PKR (Fig. 3). In vitro phosphorylation reactions carried out with His-PKR(K296R) substrate alone did not yield any detectable phosphoprotein product, either in the absence or in the presence of dsRNA (Fig. 3, lanes 7 and 8). This result provides further evidence that the K296R mutation indeed inactivates PKR kinase activity (12). However, when wt PKR was added to the reaction mixture containing His-PKR(K296R), two phosphoproteins appeared, the 67-kDa wt PKR and the 71-kDa His-PKR(K296R) (Fig. 3, lanes 9 and 10). Their phosphorylation was RNA dependent.

Inactivation of wt PKR by heat treatment completely abolished its RNA-dependent autophosphorylation (Fig. 3, lanes 3 and 4 versus lanes 1 and 2). When wt PKR was heat inactivated prior to its addition to the reaction mixture containing His-PKR(K296R), neither the 67- nor the 71-kDa phosphoprotein was observed (Fig. 3, lanes 11 and 12). The presence of 2AP significantly reduced the autophosphorylation of wt PKR, although some residual activity could be detected (Fig. 3, lanes 5 and 6). Similarly, addition of 2AP to the phosphorylation reaction mixtures containing both wt PKR and His-PKR(K296R) significantly reduced the phosphorylation of both proteins (Fig. 3, lanes 13 and 14). Thus, the phosphorylation of His-PKR(K296R) was dependent on the presence of active wt PKR, because either the inactivation of wt PKR by heat treatment or the inhibition of wt PKR by 2AP blocked the phosphorylation of both wt PKR and His-PKR(K296R) in a parallel manner.

Activation of human PKR kinase autophosphorylation by PKR mRNA. To address the question concerning the nature of the RNA that activated PKR catalytic activity in reticulocyte lysates (Fig. 1), it should be kept in mind that not only true dsRNAs, but also certain single-stranded RNAs with double-stranded regions, such as the reovirus s1 mRNA or human immunodeficiency virus TAR RNA (4, 7, 27) are potent activators of PKR. Thus, it is possible that the in vitro-transcribed PKR RNA utilized to program the reticulocyte lysate was itself capable of activating the synthesized PKR protein. Consistent with this notion, we and others previously reported that the synthesis of wt PKR in transfected monkey COS cells is autoregulated at the level of translation by a mechanism that is dependent on PKR catalytic activity (1, 29). Conceivably the RNA responsible for the activation of PKR synthesized in vivo, like that observed in vitro (Fig. 1), is PKR mRNA itself.

As shown by Fig. 4, PKR mRNA synthesized in vitro indeed was capable of mediating the activation of PKR autophosphorylation. Purified human PKR was dependent upon RNA for catalytic activity, as measured by the autophosphorylation of the 67-kDa PKR protein. This was established by using poly(rI) · poly(rC) as a positive control (Fig. 4, lanes 2 to 4). Very little PKR autophosphorylation was observed in the absence of added activator RNA (Fig. 4, lane 1). However, the 2,350-nucleotide PKR mRNA synthesized in vitro with T7 RNA polymerase activated the purified PKR kinase (Fig. 4, lanes 11 to 13). The activation properties of the PKR mRNA were more similar to those described for the 1,462-nucleotide reovirus s1 mRNA (Fig. 4, lanes 5 to 7), an established activator of PKR (4, 24), than to those described for reovirus s4 mRNA. The 1,196-nucleotide reovirus s4 mRNA was a poor kinase activator (Fig. 4, lanes 8 to 10) when examined at RNA concentrations between 0.01 and 1.0 μg/ml as previously reported (4).

Conclusions. In this report, we demonstrate definitively that the IFN-induced PKR can be autophosphorylated in an intermolecular manner. Mutant PKR(K296R) phosphorylation oc-

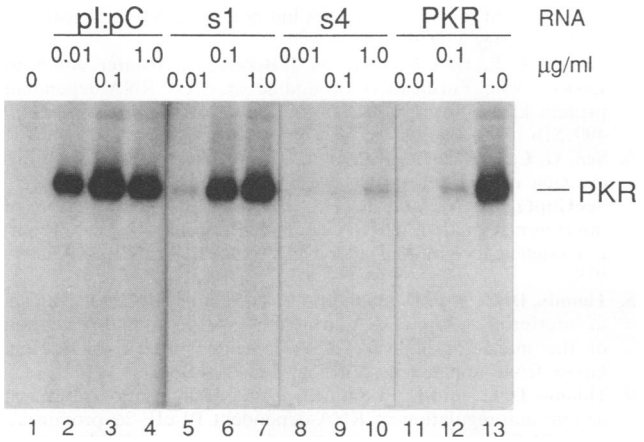

FIG. 4. Activation of wt PKR by PKR mRNA. In vitro phosphorylation reaction mixtures contained the following additions: no RNA (lane 1); poly(rI) · poly(rC) at 0.01, 0.1, and 1.0 µg/ml (pI:pC; lanes 2 to 4); s1 mRNA (s1; lanes 5 to 7); s4 mRNA (s4; lanes 8 to 10); or PKR mRNA (PKR; lanes 11 to 13). Reovirus s1 and s4 mRNAs and PKR mRNA were in vitro transcribed with T7 RNA polymerase and quantitated with ^{3}H-labeled rUTP. Reaction mixtures were incubated at 30°C for 2 min, and ^{32}P-labeled phosphoproteins were analyzed on an SDS-PAGE (10% polyacrylamide) gel followed by autoradiography. The position of the 67-kDa PKR phosphoprotein is indicated.

curred only in the presence of active wt PKR and was dependent on RNA. Because PKR(K296R) alone possessed no detectable kinase activity, it was presumably phosphorylated in *trans* by wt PKR protein via an intermolecular phosphorylation mechanism. Inactivation or inhibition of wt PKR by heat treatment or 2AP addition, respectively, dramatically reduced phosphorylation of wt PKR as well as PKR(K296R). These results strongly suggest that the mutant PKR(K296R) protein was a substrate of the wt PKR, an active kinase. The finding that RNA-dependent activation of PKR may involve intermolecular autophosphorylation events is consistent with one of the proposed models for PKR activation, in which at least two PKR molecules are required to be bound to the same dsRNA molecule for subsequent intermolecular phosphorylation of one PKR molecule by the other (14). However, the occurrence of intermolecular PKR autophosphorylation does not exclude the possibility that activation of PKR may also occur by intramolecular autophosphorylation (2, 9).

The expression of wt PKR in transfected COS cells is autoregulated primarily at the level of translation by a mechanism dependent upon catalytically active PKR kinase (29). The finding that PKR mRNA itself activates the PKR kinase provides an added parameter for localizing PKR expression and function within cells and provides an insight into the mechanism of translational autoregulation. Thus, it appears that IFN not only induces the synthesis of a key regulatory enzyme, PKR, but it also induces an activator of PKR. Such autoregulation of PKR expression could be especially important during the later stages of host recovery from viral infection when high levels of PKR protein are no longer beneficial to the cell (29). When the sites of phosphorylation of PKR have been determined by direct chemical analysis, it then will be possible to construct site-directed substitution mutants that possess either single or multiple alterations of the phosphorylation sites. Quantitative analysis of the catalytic activity of such mutants, both for PKR autophosphorylation and for eIF-2α phosphorylation, should provide further biochemical insights into the pathway of PKR activation.

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