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Activation of the double-stranded RNA (dsRNA)-activated human protein kinase *in vivo* in the absence of its dsRNA binding domain

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ABSTRACT **The interferon-induced, dsRNA-activated human protein kinase (PKR) exerts antiviral and antiproliferative effects through inhibition of protein synthesis. Studies of structure–function relationships in PKR have shown that two dsRNA binding motifs are important for its autophosphorylation and activation by dsRNA *in vitro*. To correlate these findings with the activity of PKR *in vivo*, we examined the function of various PKR deletion mutants in cultured cells by using an inducible expression system. In a reporter gene assay, mutant forms of the kinase lacking amino acids 1–97 (Δ1–97) and 104–157 (Δ104–157), which are required for dsRNA binding *in vitro*, retained full activity *in vivo*. Deletion of amino acids 233–271 (Δ233–271), however, abolished the translational inhibitory activity of the kinase and prevented its phosphorylation. Moreover, cells infected with vaccinia virus recombinants expressing wild-type PKR, the mutant Δ104–157, or a triple deletion mutant form of PKR (Δ1–97, Δ104–157, Δ186–222), developed almost complete inhibition of both viral and cellular protein synthesis upon induction of PKR. This inhibition of viral protein synthesis was not observed in cells infected with a recombinant expressing Δ233–271 mutant PKR. Our findings establish that the region encompassing amino acids 233–271 of PKR is critical for kinase activity *in vivo*, whereas its dsRNA binding domain is dispensable.**

The interferon (IFN)-induced, dsRNA-activated human protein kinase (PKR; also termed DAI, p68 kinase, dsI, PK_{ds}, and P1/eIF2α protein kinase) is thought to be a key mediator of the antiviral and antiproliferative effects of IFN (1, 2). In the presence of dsRNA and ATP, PKR is autophosphorylated on several of its serine and threonine residues (3). Autophosphorylated PKR, in turn, catalyzes the phosphorylation of the α subunit of eukaryotic initiation factor 2 (eIF-2α), which leads to inhibition of protein synthesis (4). Recent studies have demonstrated directly the antiviral function of PKR (5, 6) and its ability to suppress growth in yeast (7). In addition, PKR has been suggested to be a tumor suppressor because expression of catalytically inactive mutant forms of the kinase can cause malignant transformation (8, 9). The tumor suppressor function of PKR has also been correlated with the induction of apoptosis (10).

The conserved kinase motifs (11) are represented in the C-terminal half of PKR, while the N-terminal half of the protein, which presumably plays a regulatory role, contains three clusters of basic amino acids (aa) (12, 13). These basic regions encompass aa 1–97, 104–157, and 233–271. Studies of various mutants of PKR *in vitro* revealed the existence of a dsRNA binding domain within the first 171 aa (7, 13–17). This domain is composed of two divergent copies of an RNA binding motif, each of which contains one of the basic clusters (13). Thus, the first two basic regions, which span aa 1–97 and 104–157, are required for dsRNA binding, whereas the third basic region, aa 233–271, is dispensable for this function (13).

Comparable structure–function studies *in vivo* are hampered by the toxic effects of PKR. To overcome this limitation, we established an infection-transfection system in which expression of PKR is driven by a vaccinia virus (VV) late promoter and regulated by *Escherichia coli* lacI repressor/operator control elements (18). In this system, cultured cells are infected with VV and transfected with two plasmids, one containing the inducible PKR gene and the other carrying a luciferase (LUC) reporter gene. Production of PKR leads to reduced LUC expression. We have also developed VV recombinants in which activation of PKR leads to a severe inhibition of both viral and cellular protein synthesis during infection (6). These two systems provide the means to study the structure–function relationship of PKR *in vivo*. In this investigation we have analyzed the activity of deletion mutants of PKR using both the VV infection-transfection system and the VV recombinant system. We report that the third basic region, which spans aa 233–271, is critical for the activation of PKR. Moreover, we show that activation of PKR can occur in the absence of both dsRNA binding motifs (aa 1–97 and aa 104–157). These results suggest that the endogenous PKR may phosphorylate the transfected kinase and that the third basic region is required for this activation.

MATERIALS AND METHODS

Construction of VV Insertional Vectors Containing Mutant Forms of PKR. Plasmids pTL1 and pTL11 containing the cDNAs encoding the entire human PKR gene (wild-type and lys296→arg mutant, respectively) have been described (18). The pTL constructs are based on a VV insertional vector, pPR35, for inducible expression (19). It consists of the following genetic elements: the VV late promoter p4b, two 21-bp lac operators (op), and the lac repressor gene under control of the VV early-late promoter p7.5 (Fig. 1). The cassette is flanked at both ends with VV thymidine kinase sequences to provide sites of insertion into the VV genome. A schematic representation of wild-type and deletion mutants of PKR is shown in Fig. 1. Plasmid pTL1 was digested with *Bam*HI and *Nco* I, the ends were filled with Klenow fragment, and the vector was self-ligated to generate pTL12, containing a deletion of aa 1–97 (Δ1–97), equivalent to Δ1 (13). Plasmids Δ2 and Δ3 (13) harboring deletions of aa 104–157 and 233–271, respectively, were treated with *Sty* I to excise the fragments that contain the deletions, and the

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Abbreviations: IPTG, isopropyl β-D-thiogalactoside; VV, vaccinia virus; p.i., postinfection; pfu, plaque-forming units; dsRNA, double-stranded RNA; PKR, dsRNA-activated human protein kinase; LUC, luciferase.

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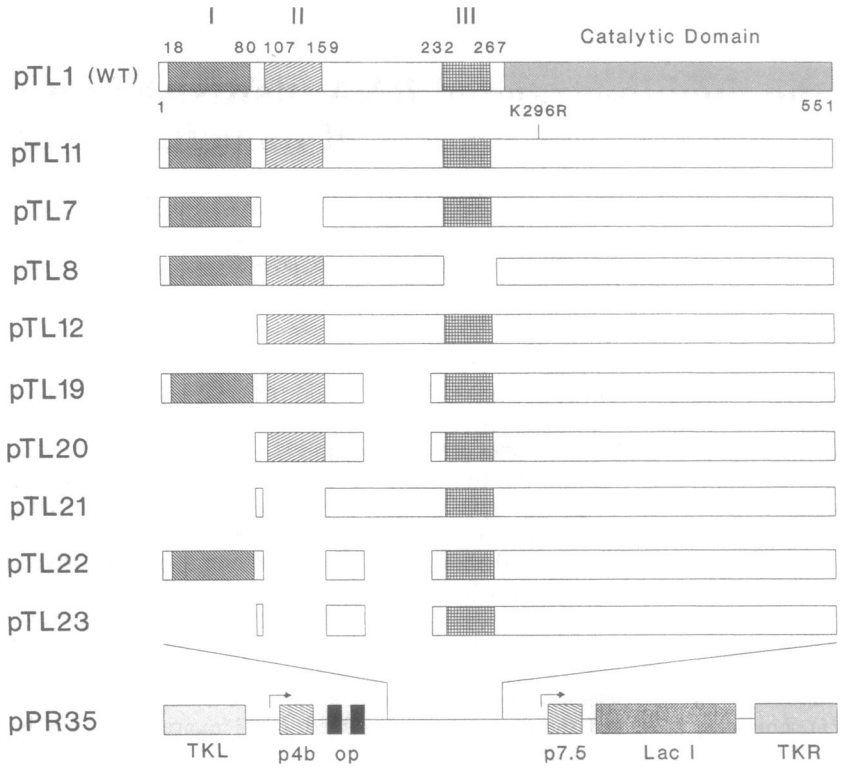

FIG. 1. Schematic representation of inducible insertional VV vector pPR35 (19) and different deletion mutants of PKR. TKL, VV thymidine kinase gene, left end; TKR, VV thymidine kinase gene, right end; p4b, VV late promoter; op, lac operator; p7.5, VV early-late promoter; Lac I, lac repressor. Arrows indicate the direction of gene expression. Hatched boxes I, II, and III indicate three regions that are rich in basic amino acids within the N terminus of PKR. The numbers above the hatched boxes denote the amino acid residues. The catalytic domain is comprised of 270 aa of the C terminus of PKR and is shown in the dotted box. K296R indicates a single point mutation, which converts the lysine residue at aa 296 to arginine. WT, wild-type PKR.

resultant fragments were inserted between equivalent sites in pTL1 to generate pTL7 and pTL8, respectively. Plasmid pTL1 was digested with *Bsa*AI and *Pme* I and self-ligated to yield pTL19 containing a deletion of aa 186–222. The cloning strategy used for pTL12 was used to generate the double deletions pTL20 (Δ1–97, Δ186–222) and pTL21 (Δ1–97, Δ104–157) from pTL19 and pTL7, respectively. The strategy used for pTL19 was applied to pTL7 to yield pTL22 (Δ104–157, Δ186–222). Plasmid pTL22 was further treated in the same manner as used to generate pTL12 to yield pTL23 containing deletions from aa 1–97, 104–157, and 186–222. All constructs were confirmed by DNA restriction analysis.

Growth of Cells and Viruses. Monolayer cultures of African green monkey kidney BSC-40 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% newborn calf serum (GIBCO/BRL). The Western Reserve (WR) strain of VV and VV recombinants were grown and prepared as described (20).

Generation of VV Recombinants Expressing Mutant Forms of PKR. Recombinant VVs expressing the wild-type or lys296→arg PKR have been described (6). Viruses expressing the deletion mutants Δ104–157 (WR68KΔ2), Δ233–271 (WR68KΔ3), and Δ1–97, Δ104–157, Δ186–222 (WR68KΔTri) were generated using the pTL7, pTL8, and pTL23 plasmids, respectively, according to the procedure described previously (21). Recombinant viruses were selected with 5′-bromodeoxyuridine, and two rounds of plaque purification yielded homogeneous virus stocks. The PKR insertion site in the VV genome was confirmed by Southern blot analysis using total DNA from infected cells.

Infection-Transfection Analysis. BSC-40 cells were infected for 1 hr with VV at 2 plaque-forming units (pfu) per cell. After removing the inoculum, the cells were transfected (Lipofect-ACE; BRL) with plasmids expressing PKR for 3 hr and then incubated with fresh DMEM containing 2% newborn calf serum either with or without 1.5 mM isopropyl β-D-thiogalactoside (IPTG) for 18 hr.

Immunoprecipitation Analysis. BSC-40 cells grown in 60-mm plates were infected at 2 pfu per cell with VV and transfected with 10 μg of indicated plasmids in the absence or in the presence of 1.5 mM IPTG. At 20 hr postinfection (p.i.) cells were metabolically labeled for 3 hr with 50 μCi of [^{32}P]orthophosphate per ml (1 Ci = 37 GBq) in phosphate-free medium. Cell extracts were prepared and immunoprecipitated as described (6), using rabbit polyclonal antibody specific for human PKR (13) [1:50 dilution in phosphate-buffered saline (PBS) with 5% Blotto (nonfat dry milk)]. Immunoprecipitates were analyzed by 8% SDS/PAGE and autoradiography.

Measurement of LUC Activity. BSC-40 cells grown in 12-well Limbro plates were infected at 2 pfu per cell with VV and cotransfected with 1 μg of PKR plasmid together with 1 μg of pPR15 (19), with or without 1.5 mM IPTG. Plasmid pPR15 contains the LUC reporter gene under control of the VV p4b late promoter, the same promoter that drives the expression of the PKR constructs but without the lac repressor/operator element. LUC activity in cell extracts was measured at 24 hr p.i. (22, 23).

Immunoblot Analysis. For transient infection-transfection expression of PKR, BSC-40 cells grown in 12-well Limbro plates were infected at 2 pfu per cell with VV and transfected with 2 μg of PKR plasmid, with or without 1.5 mM IPTG. At 24 hr p.i., cells were washed twice with PBS and extracts were collected in 1× SDS-reducing sample buffer (31 mM Tris·HCl, pH 6.8/2% SDS/5% 2-mercaptoethanol/5% glycerol/0.01% bromophenol blue). Proteins were separated by 8% SDS/PAGE, transferred to nitrocellulose membrane, and allowed to react with mouse polyclonal antibodies against

PKR (18) (1:250 dilution in PBS with 5% Blotto) followed by immunoperoxidase staining. For recombinant virus studies, BSC-40 cells grown in 12-well plates were mock-infected or infected at 5 pfu per cell with recombinant VV in the absence or presence of 1.5 mM IPTG. Cell extracts were collected at 24 hr p.i. and analysis of PKR was done as described above. To detect VV proteins, identical samples were separated by 10% SDS/PAGE, transferred, and allowed to react with rabbit polyclonal antibodies against VV (1:1000 dilution in PBS with 5% Blotto).

RESULTS

Expression and Phosphorylation of Mutant PKR in Transfected Cells. The protein kinase PKR is activated by dsRNA in a reaction that is accompanied by autophosphorylation. We demonstrated previously that induction of wild-type PKR in transfected cells leads to inhibition of cotransfected reporter gene expression (18). This inhibition requires enzymatically active PKR and thus provides a convenient system in which to study the structure–function relationship of PKR *in vivo*. To this end, we generated a panel of plasmids expressing various deletion mutants of PKR under the control of an inducible promoter (Fig. 1). The mutants contained single, double, or triple deletions within the N-terminal half of the kinase, spanning its dsRNA binding domain as well as other regions, but sparing the conserved kinase domain. To examine the expression and autophosphorylation of PKR, BSC-40 cells were infected with VV and transfected with plasmids containing different deletion mutants of the protein kinase.

After induction of the kinase with IPTG and metabolic labeling with [^{32}P]orthophosphate, cell extracts were immunoprecipitated with rabbit polyclonal antibodies against human PKR (13). SDS/PAGE analysis of the immunoprecipitates revealed different sizes of activated human PKRs as well as background phosphorylation of the endogenous monkey kinase, p69 (Fig. 2*A*). As previously described (18), we detected an increased phosphorylation of wild-type PKR with the addition of IPTG. Deletion of either the second basic region (aa 104–157) or the region between the second and third basic regions (aa 186–222) had no effect on PKR phosphorylation (lanes 7 and 8 and lanes 11 and 12). Surprisingly, however, autophosphorylation of the deletion mutant lacking the third basic region (aa 233–271) was greatly reduced (lanes 9 and 10). The catalytically inactive lys296→arg mutant was also poorly phosphorylated (lanes 5 and 6), as reported previously (6). A phosphoprotein of about 43 kDa was coimmunoprecipitated; it probably represents a protein related to PKR such as its breakdown product (24). Furthermore, double deletion (Δ104–157, Δ186–222) and triple deletion (Δ1–97, Δ104–157, Δ186–222) mutants of PKR, which lack either one or both of the dsRNA binding motifs but retain residues 233–271, were still phosphorylated (lanes 13–16), suggesting that the third basic region (aa 233–271) is critical for autophosphorylation of PKR. As shown by Western blot analysis (Fig. 2*B*), the lack of phosphorylation of Δ233–271 or lys296→arg PKR mutants cannot be attributed to instability of these kinases. In addition, the degree of induction of PKR proteins seems to be inversely related to their activity, presumably because PKR down-regulates its own synthesis (6, 25).

Inhibition of Translation by Mutant Forms of PKR. To assess the ability of the deletion mutants to inhibit protein synthesis, we cotransfected the kinase vectors with a plasmid carrying the reporter gene, LUC. Inhibition of LUC activity is a reflection of inhibition of protein synthesis and not of transcriptional regulation (18). As shown in Fig. 3, upon induction with IPTG most of the deletion mutants inhibited LUC activity to a similar extent as the wild-type PKR

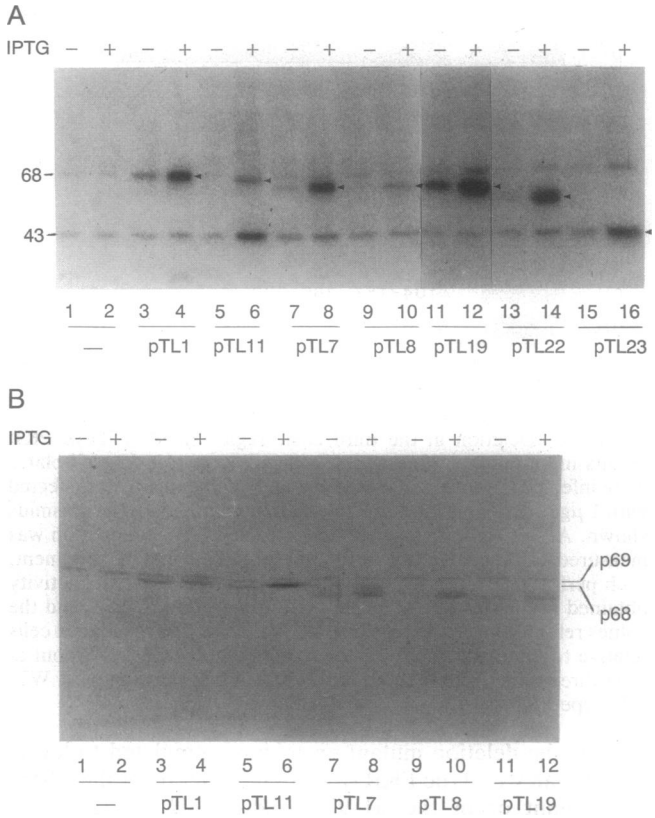

FIG. 2. Expression and autophosphorylation of deletion mutants of PKR *in vivo*. (*A*) Immunoprecipitation analysis. BSC-40 cells grown in 60-mm plates were infected with 2 pfu per cell of wild-type VV and independently transfected with 10 μg of mutant PKR plasmids specified. At 20 hr p.i., cells were metabolically labeled with [^{32}P]orthophosphate for 3 hr. Cell extracts were immunoprecipitated with rabbit polyclonal antibody against PKR (13) and analyzed by 8% SDS/PAGE followed by autoradiography. Arrowheads denote transfected mutants or wild-type PKR. Molecular mass markers in kDa are shown to the left. (*B*) Western blot analysis. BSC-40 cells, after infection-transfection, were lysed at 24 hr p.i. and extracts were analyzed by 8% SDS/PAGE followed by immunoblotting with mouse polyclonal antibodies specific for PKR (18). Lanes 1 and 2, VV-infected cells that were not transfected with any plasmid.

(80–85%). The mutant that lacks the second basic region (Δ104–157) was less active, but the triple mutant, which lacks almost all of the N-terminal half of the protein except for the third basic region, was essentially as active as wild-type PKR. The only deletion mutant that was inactive in terms of inhibiting the levels of LUC was the deletion mutant that lacked the third basic region, Δ233–271. The catalytically inactive mutant, lys296→arg, also did not inhibit LUC activity as expected (18). These results show that the third basic region, aa 233–271, is important for the activation of PKR and that activation of PKR can occur in the absence of both of the dsRNA binding motifs, aa 1–97 and 104–157.

Expression of Δ233–271 Mutant PKR Fails To Inhibit Viral Protein Synthesis. The observation that a mutant PKR that lacks both of its dsRNA motifs can still be active was very surprising; therefore, we needed to confirm the ability of deletion mutants to inhibit virus protein synthesis *in vivo*. For this purpose, we generated three VV recombinants expressing mutant PKR lacking the second basic region (Δ104–157), the third basic region (Δ233–271), or the entire N-terminal half of the kinase except the third basic region (Δ1–97, Δ104–157, Δ186–222). Immunoblot analysis of lysates of cells infected with these VV recombinants demonstrated that the addition of IPTG induced expression of PKR and that the deletion mutants of PKR are as stable as the full-length PKR.

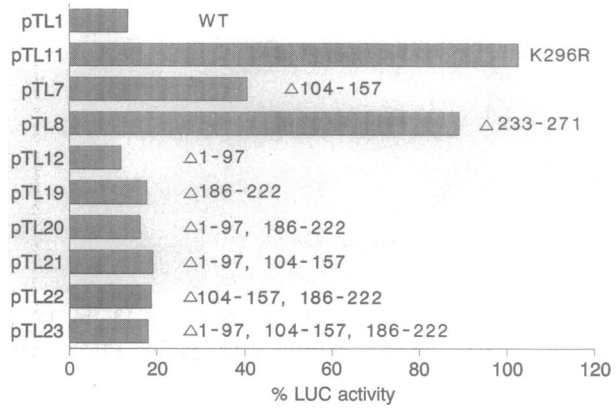

FIG. 3. Deletion in the third basic region (Δ233–271) of PKR results in an inactive kinase. BSC-40 cells grown in 12-well plates were infected (2 pfu per cell) with VV and independently transfected with 1 μg of LUC plasmid pPR15 together with 1 μg of the plasmids shown. At 24 hr p.i., cells were collected and LUC production was measured. Average LUC activity measured in a single experiment, each performed in duplicate, is shown. In all assays, LUC activity obtained in the absence of IPTG was normalized to 100%, and the values refer to the percentage of LUC activity found in induced cells relative to uninduced cells. These experiments were carried out at least three times with <10% variation seen between samples. WT, wild-type PKR.

In fact, the deletion mutant proteins accumulated to levels higher than wild-type PKR (Fig. 4*A*). Induction of the Δ104–157 mutant or of the Δ1–97, Δ104–157, Δ186–222 triple deletion mutant resulted in a severe inhibition of viral protein synthesis in cultured cells, to a similar extent as the wild-type PKR (Fig. 4*B*, lanes 11–14). In cells infected with a VV recombinant expressing Δ233–271 mutant PKR (Fig. 4*B*, lanes 9 and 10), however, the level of viral protein synthesis was the same as that in cells infected with wild-type virus lacking the PKR gene (Fig. 4*B*, lanes 3 and 4) or with the recombinant virus carrying the gene for the catalytically inactive lys296→arg mutant (Fig. 4*B*, lanes 7 and 8). These results are in accordance with the LUC data (Fig. 3) and reinforce the validity of the transient infection-transfection assay in assessing the activity of PKR. Virus yields were also not affected in cells infected with VV recombinant, WR68KΔ3, while VV production was severely reduced in cells infected with VV recombinants, WR68KΔ2 and WR68KΔTri (data not shown). Thus, the third basic region, but not the first or second, is required for inhibition of viral protein synthesis.

DISCUSSION

In this report we have used two systems to conduct a structure–function analysis of the human PKR, one based on transient infection-transfection and the other based on VV recombinants. In both systems, expression of various deletion mutants of PKR can be induced with IPTG in cultured cells. The major advantage of the transient infection-transfection system is that, by using a reporter gene, it allows us to conveniently measure the activity of the transfected PKR gene, while with VV recombinants we measure directly inhibition of virus protein synthesis during infection. Because of the toxicity of PKR, *in vivo* structure–function studies of the kinase have been limited to date. In one of these studies, Feng *et al.* (17) used a yeast cell growth inhibition assay to show that PKR deletions between aa 39 and 50 and aa 58 and 69 eliminated the slow-growth phenotype caused by expression of wild-type PKR. Other studies examined the ability of PKR to inhibit its own synthesis in transfected COS cells (25–27). Thomis and Samuel (25) showed that the catalytically inactive lys296→arg mutant kinase was expressed at about 30-fold higher levels than wild-type PKR, and similar observations were presented by Barber *et al.* (26) and confirmed here (Figs. 2*B* and 4*A*). Deletion of aa 156–243 had little effect on the kinase, but removal of aa 91–243 led to a 20- to 25-fold reduction of kinase activity and a 70-fold increase in its expression (26). Interestingly, a point mutation that converted lysine-64 to glutamic acid abolished the RNA binding activity and resulted in a 15-fold increase in its expression (27). Based on these and other *in vitro* studies (7, 13–17), one would expect that deletions of either the first (aa 1–97) or the second (aa 104–157) dsRNA binding motifs would be deleterious to kinase activity in tissue culture cells. Surprisingly, however, we found that deletions in both dsRNA binding motifs (aa 1–97 and aa 104–157), either separately or in combination, were permitted without affecting the ability of PKR to inhibit expression of a reporter gene or virus genes in our system. In fact, the only requirement for the activation of PKR in our system was the presence of the third basic region (aa 233–271), which lies outside the RNA binding domain.

Why is the third basic region important for PKR function? The sequence of this region does not offer any obvious clues, and it is possible that it serves some structural role in maintaining enzyme conformation that will become evident

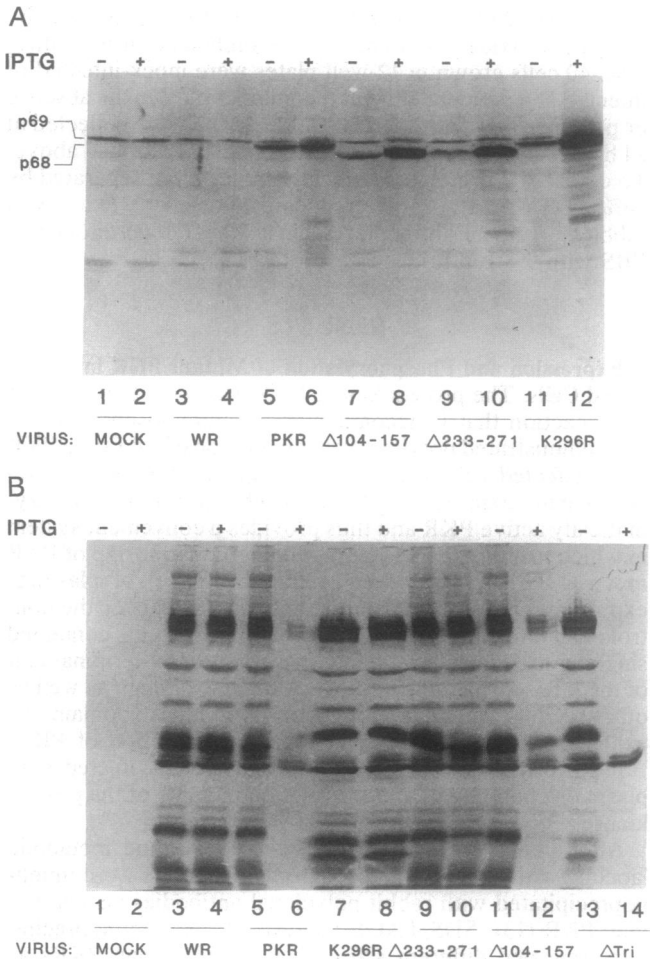

FIG. 4. Effects of PKR mutants on viral protein synthesis. BSC-40 cells were mock-infected or infected at 5 pfu per cell with different recombinant VVs, and cell extracts were collected at 24 hr p.i. (*A*) Proteins were resolved by 8% SDS/PAGE, transferred to nitrocellulose membrane, and allowed to react with mouse polyclonal antibodies specific for PKR (18). (*B*) Proteins were resolved by 10% SDS/PAGE, transferred, and allowed to react with rabbit polyclonal antibodies against VV.

only when the enzyme's three-dimensional structure is known. Based on secondary structure predictions, this region may adopt a helical form, raising the possibility that it is equivalent to the A helix, which is contiguous to the catalytic core of several protein kinases (28). However, we do not think this is very likely for two reasons. (*i*) The A helix is important for stability but not for function (28), whereas the third basic region of PKR is important for function but probably not for stability. (*ii*) It lacks the diagnostic tryptophan that seems to play an important part in A helix interactions with the kinase core (28). An alternative hypothesis is suggested by the observation that its removal greatly reduces phosphorylation, indicating that the region contains sequences important for this activity, or the phosphorylation sites themselves. The finding of several phosphorylation sites in this region supports the latter possibility, but major sites are also present elsewhere in the protein (D. Taylor and M.B.M., unpublished observations).

How can the enzyme become active in the absence of its RNA binding domain? One possibility is that deletions in either or both dsRNA binding motifs (aa 1–97 and aa 104–157) render the kinase constitutively active. We demonstrated previously that in cells infected with a recombinant virus expressing wild-type PKR, inhibition of protein synthesis does not occur before 6 hr p.i.; furthermore, the kinase is not phosphorylated between 2 and 5 hr p.i. despite the fact that the maximal synthesis of PKR was occurring at 5 hr p.i. (6). This suggests that wild-type PKR is not constitutively active in our system, requiring accumulation of an activator. When cells were infected with a virus expressing the deletion mutant PKR, which lacks both of its dsRNA binding motifs (WR68KΔTri), inhibition of protein synthesis also did not occur before 6 hr p.i. and the mutant PKR was not phosphorylated before 5 hr p.i. (data not shown). These observations argue against the idea that deletions of both dsRNA binding motifs result in constitutively active kinase. Moreover, the isolated kinase domain of PKR does not autophosphorylate or phosphorylate eIF-2α *in vitro* (S.R.G. and M.B.M., unpublished observations). A second possibility is that the endogenous monkey cell PKR phosphorylates and activates the mutant kinases, provided they are not enzymatically crippled. On this basis, mutants in the RNA binding domain could be activated, whereas the lys296→arg and Δ233–271 mutants would be refractory. Although this would seem to pose a problem in regulating the enzyme by generating all-or-none activation, the trans-phosphorylation of inactive PKR variants by the active enzyme has recently been obtained (ref. 29; C. Schmedt, S.R.G., D. Taylor, and M.B.M., unpublished observations). Finally, there may be endogenous activators other than dsRNA that function *in vivo*. It has been assumed that dsRNA is the physiological activator of PKR, but direct proof is scarce and heparin and other molecules with polyanionic structure have been shown to activate PKR *in vitro* (30). Our study indicates that activator(s) other than dsRNA should be considered. Identifying the nature of these activators should shed some light on the physiological functions of PKR.

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