JOURNAL OF VIROLOGY, Nov. 1996, p. 8128–8132 Vol. 70, No. 11
0022-538X/96/$04.00+0

Possible Involvement of Double-Stranded RNA-Activated Protein Kinase in Cell Death by Influenza Virus Infection

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Received 4 March 1996/Accepted 6 August 1996

We reported previously that influenza virus infection induces the apoptotic death of HeLa cells associated with activation of the Fas gene. In this report, we show that transfection with a PKR having a point mutation in the catalytic domain of K at 296 to R suppressed both the augmented expression of Fas and cell death by influenza virus infection. These results suggested the involvement of PKR in influenza virus-induced cell death.

The influenza virus has a genome consisting of eight negative, single-stranded RNA segments. This virus causes worldwide epidemics of acute upper respiratory tract inflammation and often complicates other conditions, particularly in the elderly. However, the mechanisms involved in damage to host cells have not been clarified. Takizawa et al. (28) and others (5, 7, 21) have reported that influenza virus infection induces apoptotic death in vitro and in mice in vivo. We also found that virus infection activated the Fas/Apo-1 (CD95) gene (32), the product of which is a transmembrane protein (8) that induces a cytolytic signal in cells upon binding to a natural ligand (26). Some other viruses also augment Fas expression (31) or increase the sensitivity of host cells to agonistic anti-Fas monoclonal antibody (MAb) (10, 16), suggesting a crucial role of Fas in the selective deletion of tissues or cells. We suggested that double-stranded RNA (dsRNA)-activated protein kinase (PKR) is involved in the induction of Fas by virus infection, since a synthetic dsRNA, poly(I)-poly(C), similarly increased the amount of Fas mRNA which was inhibited by the potent PKR inhibitor 2-aminopurine and since elimination of beta interferon from the culture medium by the antibody decreased the PKR activity as well as the induction of Fas mRNA by dsRNA (27).

PKR is induced by interferons, and it is thought to participate in their antiviral activity (24). PKR overexpression also inhibits the growth of *Saccharomyces cerevisiae* (3) and induces apoptosis in HeLa cells (14). On the other hand, the expression of a catalytically inactive mutant of PKR or mutants with a deleted RNA-binding domain confers transformation phenotypes upon NIH 3T3 cells, suggesting that PKR functions as a tumor suppressor (1, 11, 18). However, the mechanisms by which PKR acts as a tumor suppressor remain unknown. Transgenic mice devoid of PKR activity have been generated and tumors have not spontaneously arisen (34), arguing against PKR being a tumor suppressor. In this study, we showed that the expression of a catalytically inactive PKR mutant in HeLa cells transdominantly suppresses the augmented expression of Fas upon influenza virus infection and confers resistance to the cell killing effect of the virus or anti-Fas MAb. These results suggested that PKR plays an essential role in the apoptotic pathway.

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We introduced a mutant PKR cDNA having the amino acid K at 296 replaced with R (PKR K/R) into HeLa cells. This mutation does not have catalytic activity (9). The mutant PKR cDNA was constructed by sequential PCR (4). The primers PKR-1 (5′-ACGTTATTAGACGTGTTA-3′; nucleotides 1064 to 1081 of the human PKR cDNA [17]), PKR-2 (complementary to PKR-1; 1081 to 1065), PKR-3 (5′-ACAACGAATTCT TCAGAAGG-3′; 451 to 470), and PKR-4 (5′-TCACAGAAT TCCATTTGGAT-3′; 1295 to 1276) were synthesized. PKR-1 and PKR-2 contain one mutated nucleotide at 1073 that converts the K (AAA) at 296 to R (AGA). Initially, we amplified a portion of PKR cDNA using PKR-3 and PKR-2 or PKR-1 and PKR-4. The resulting two DNA fragments were annealed, and secondary PCR was performed with primers PKR-3 and PKR-4. The amplified product was digested with *Nco*I and *Eco*RI, and then the fragment was inserted into the corresponding region of the wild-type PKR cDNA. Mutation was verified by DNA sequencing by dideoxy chain termination (25). Wild-type and mutant PKR cDNAs were then inserted into the multicloning site of the pRC-CMV vector (Invitrogen). HeLa cells were transfected with 2 μg of plasmid DNA per 5×10^5 cells by using 10 μl of Lipofectin reagent (Gibco BRL). To establish permanent transfectants, cells were diluted about 10-fold and replated in medium containing 500 μg of G418 per ml 2 days after transfection and then drug-resistant colonies were isolated. We could not permanently transfect clones with wild-type PKR.

Reverse transcription-PCR (RT-PCR) was performed to verify that the mRNA encoded the mutant PKR in the cells transfected with plasmid DNA (Fig. 1A). PCR products were separated on a 2% agarose gel and stained with ethidium bromide. RT-PCR using primers PKR-3 and PKR-4 at an annealing temperature of 48°C for 50 cycles generated a DNA fragment corresponding to an 845-bp fragment of PKR cDNA both in control cells and in those transfected with mutant PKR (Fig. 1A, lanes 4 to 6). A second PCR amplification was done with 1/50 of the RT-PCR products and primers PKR-1 and PKR-4 at an annealing temperature of 52°C for 15 cycles. PCR performed under these conditions amplified only the DNA fragment from the plasmid construct of PKR K/R (Fig. 1A, lanes 1 to 3). The second PCR yielded the 231-bp DNA fragment from the RT-PCR product of mutant-PKR-transfected cells but essentially not that of the control (Fig. 1A, lanes 7 to 9), confirming the presence of mutant PKR mRNA in these cells. We then examined the kinase activities in the mutant-PKR-transfected cells after influenza virus infection. HeLa

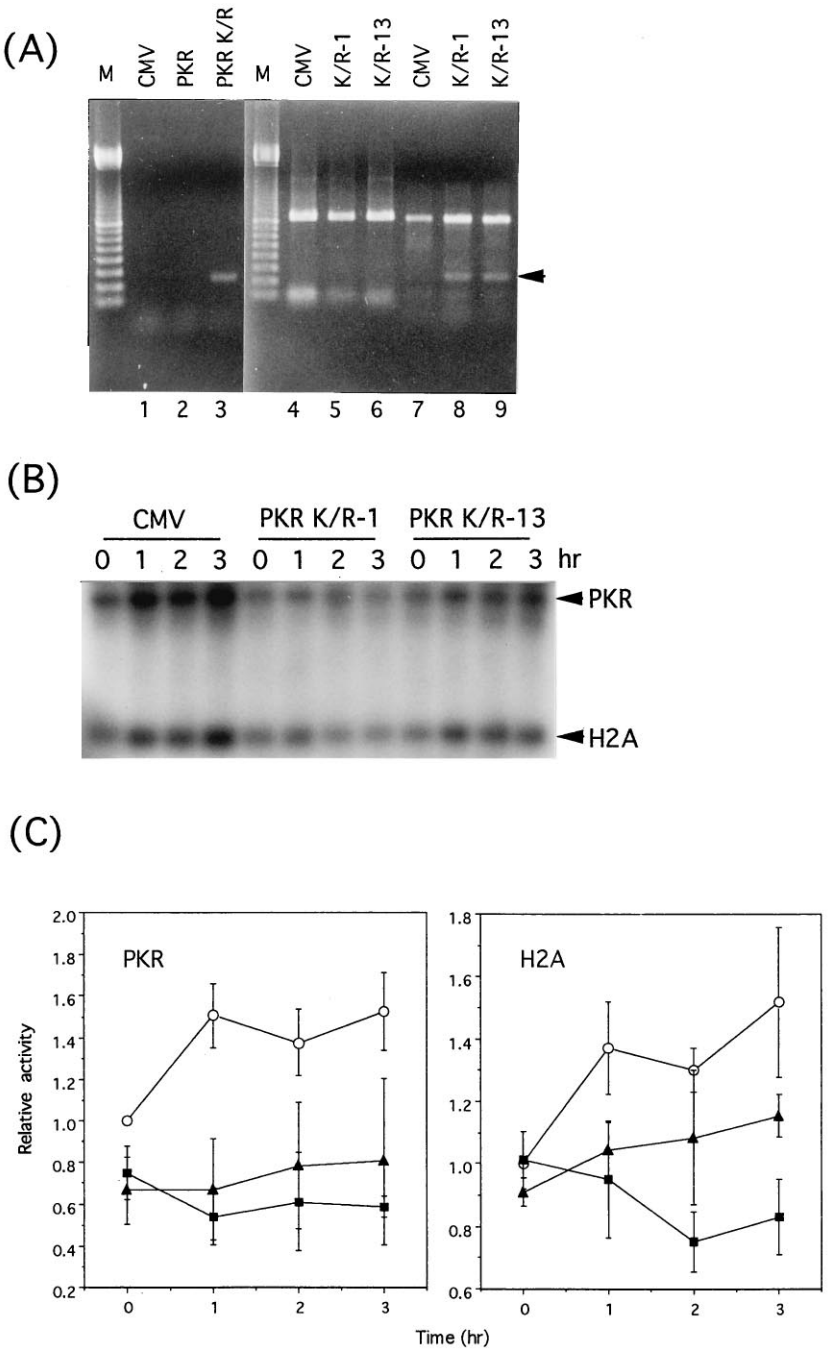

FIG. 1. Expression of mutant PKR in HeLa cells and its effect on PKR activity after influenza virus infection. (A) Detection of mutant PKR mRNA. PCR with primers PKR-1 and PKR-4 under the conditions described in the text amplified a DNA fragment from plasmid construct PKR K/R but hardly from a cytomegalovirus (CMV) vector or wild-type PKR (lanes 1 to 3). RT-PCR amplification of a PKR cDNA fragment (845-bp) was first performed by using primers PKR-3 and PKR-4 with total RNAs (2 μg) extracted from the plasmid DNA-transfected HeLa cells as indicated above lanes 4 to 6. The mutant PKR DNA (arrowhead) was then amplified from 1/50 of the RT-PCR products of lanes 4 to 6 by using primers PKR-1 and PKR-4 under the same conditions as for lanes 1 to 3. PCR products were separated on a 2% agarose gel and stained with ethidium bromide. Lane M contains a DNA ladder of 100 bp. (B) HeLa cells permanently transfected with the vector (CMV) or mutant PKR were infected with the influenza virus, and cell extracts were prepared at the times indicated above the lanes. PKR activities were assayed as described in the text. (C) The relative PKR activity was determined by scanning the autoradiogram, of which a representative is shown in panel B; activity is given as the ratio to the activity in the CMV vector-transfected cells at 0 h. Data are the means ± standard deviations of three experiments. ○, CMV-3; ■, PKR K/R-1; ▲, PKR K/R-13.

cells were infected with SP626, a wild-type strain of the influenza A/Udorn/72 (H3N2) virus, at 5 PFU per cell as described previously (28). PKR in the cell extract was precipitated by incubation with the anti-PKR MAb followed by protein G-Sepharose 4FF (Pharmacia LKB Biotechnology, Uppsala, Sweden) as described previously (6). Immunoprecipitated PKR was incubated with [γ-^{32}P]ATP (650 Ci/mmol; ICN Biomedicals, Irvine, Calif.) for 15 min at 30°C and then with 5 μg of histone H2A (Boehringer Mannheim), which is an effective substrate for PKR (6), for another 15 min at 30°C. The phos-

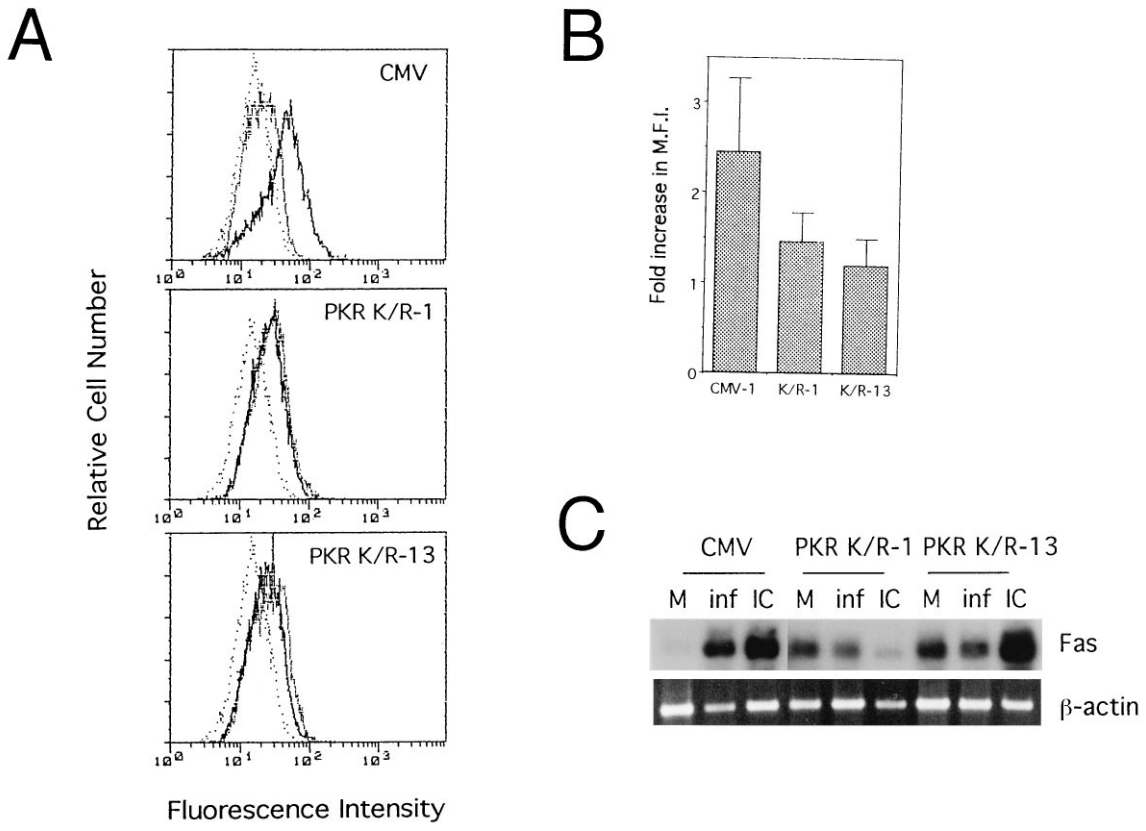

FIG. 2. Expression of Fas on the mutant-PKR-transfected HeLa cells after influenza virus infection. (A) HeLa cells transfected with the vector (cytomegalovirus [CMV]) or mutant PKR were mock infected (interrupted line) or infected with the virus (solid line) and stained 12 h postinfection with anti-Fas MAb (IgG) and then incubated with fluorescein isothiocyanate-conjugated goat anti-mouse IgG MAb. Fas on the cell surface was then analyzed by flow cytometry. The dotted line indicates a control with irrelevant antibodies. (B) The ratio of relative mean fluorescence intensities (M.F.I.) (mean fluorescent intensities minus irrelevant fluorescent intensities) between mock- and virus-infected cells is shown. Data are the means ± standard deviations of three experiments. (C) Amount of Fas mRNA in the mutant-PKR-transfected HeLa cells after influenza virus infection or poly(I)-poly(C) exposure. Total RNA was extracted from HeLa cells transfected with the vector (CMV) or mutant PKR 4 h after mock (M) or influenza virus (inf) infection or 6 h after exposure to poly(I)-poly(C) (50 μg/ml) (IC). The Fas mRNA level was determined by RT-PCR followed by Southern blotting with human Fas cDNA as the probe. The control was β-actin cDNA from the same sample amplified by RT-PCR and resolved by agarose gel electrophoresis.

phorylation reaction products were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and visualized by autoradiography. Relative PKR activity was determined by scanning the autoradiogram and is given as the ratio to the activity in the vector-transfected cells at 0 h. Since a high concentration of dsRNA seemed to mask changes in the kinase activity (data not shown), we measured it in the absence of poly(I)-poly(C). While both autophosphorylation and H2A phosphorylation activities in the extracts from the vector-transfected cells were increased about 1.5-fold 1 h after infection and later, there was either a decrease or only a slight increase in those from the mutant-PKR-transfected cells (Fig. 1B and C). Thus, PKR activation after virus infection was suppressed in the clones with mutant PKR. The level of PKR activities measured in the absence of dsRNA was much lower than that in the presence of dsRNA. Therefore, most of the kinase may be inactive even in the control cells infected with the virus. This might be due to the presence of an influenza virus-activated cellular PKR inhibitor (15).

Fas expression in the mutant-PKR-transfected HeLa cells after virus infection was examined by fluorescence-activated cell sorter analysis. Mock- or virus-infected cells were harvested and fixed with 1% paraformaldehyde as described previously (27). The cells were incubated with immunoglobulin G (IgG) anti-Fas MAb and then exposed to fluorescein isothiocyanate conjugated goat anti-mouse IgG Ab. The stained cells were analyzed on a FACStar (Becton Dickinson, San Jose, Calif.), using a single 488-nm argon laser as described previously (20). The augmented Fas staining upon virus infection was effectively suppressed in the cells permanently transfected with the mutant PKR, whereas there was about a 2.5-fold increase in mean fluorescence intensity in the vector-transfected cells (Fig. 2A and B). The background Fas staining in the mutant-PKR-transfected cells with mock infection was higher than that in the cells transfected with the vector (about a twofold increase in mean fluorescence intensity) (Fig. 2A), which may reflect either an increase in the basal amount of Fas mRNA (Fig. 2C) or enhanced protein synthesis due to the increased availability of dephosphorylated eIF-2α.

The amount of Fas mRNA was measured by RT-PCR combined with Southern blotting as described previously (27). The antisense and sense oligonucleotide primers for amplifying human Fas cDNA corresponded to nucleotides 808 to 789 (5′-TCCTTTCTGTGCTTTCTGCA-3′) and 266 to 285 (5′-TGCCCAAGTGACTGACATCA-3′), respectively (8). The antisense and sense primers for human β-actin corresponded to nucleotides 922 to 903 (5′-TACAGGTCTTTGCGGATGTC-3′) 274 to 293 (5′-ACTGGGACGACATGGAGAAA-3′), respectively (23). A portion of the amplified products was separated by electrophoresis on a 1.5% agarose gel. The increase in Fas

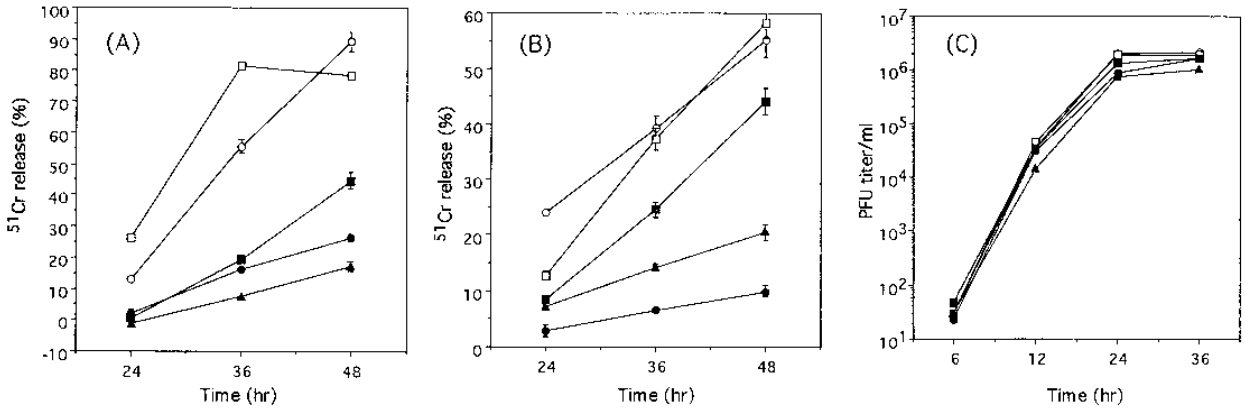

FIG. 3. Time course of the death of HeLa cells transfected with the vector (CMV-1 and CMV-3) or mutant PKR after influenza virus infection (A) or exposure to agonistic anti-Fas MAb (IgM) and poly(I)-poly(C) (B). HeLa cells were labeled with $Na_2{}^{51}CrO_4$ and infected with the virus or incubated in the presence of both anti-Fas MAb (CH-11) (50 ng/ml) and poly(I)-poly(C) (50 μg/ml). Cytolysis was determined as the percentage of specific ^{51}Cr release from the cells at various times. The percentage of specific ^{51}Cr release was determined by using the following formula: [(experimental release − spontaneous release)/(total release − spontaneous release)] × 100, where cpm is counts per minute. Data are the means ± standard deviations of three experiments except for CMV-3 in panel A, for which the means of two experiments (without error bars) are shown. Some error bars are obscured by the symbols. (C) Time course for infectious-virus titers in the medium of HeLa cells transfected with the vector (CMV-1 and CMV-3) or with mutant PKR after influenza virus infection. Virus titers were determined by measuring the PFU per milliliter on MDCK cells. Data are the means of three experiments. ○, CMV-1; □, CMV-3; ■, PKR K/R-1; ●, PKR K/R-3; ▲, PKR K/R-13.

mRNA level by the virus infection was effectively suppressed by permanent transfection with the mutant PKR but less effectively in poly(I)-poly(C)-treated cells, which contained excess dsRNA (Fig. 2C). The basal level of Fas mRNA was much higher in the cells transfected with mutant PKR than with the vector. This may be due to Fas mRNA stabilization, since the transient transfection of wild-type PKR degraded host mRNA upon virus infection or poly(I)-poly(C) exposure (data not shown). These results indicated that transfection with the mutant PKR transdominantly suppressed the augmented expression of Fas upon influenza virus infection at the mRNA level. These results were consistent with our findings that virus infection activates transcription of the Fas gene (32).

We examined the effect of mutant PKR on cell death caused by influenza virus infection. Cell viability was determined by the ^{51}Cr release assay as described previously (28). In cells transfected with mutant PKR, the progression of cell death after influenza virus infection was apparently slower than that in vector-transfected lines (Fig. 3A). We showed that poly(I)-poly(C)-treated HeLa cells were highly susceptible to agonistic anti-Fas MAb (27). The cell death caused by poly(I)-poly(C) and anti-Fas MAb was also suppressed in the mutant-PKR-transfected cell lines, although to lesser extent (Fig. 3B). This may be because poly(I)-poly(C), a potent interferon inducer, augmented the level of endogenous PKR. To examine whether viral replication was suppressed in cells transfected with mutant PKR, we measured the virus titers in the medium (Fig. 3C). Virus PFU were titrated on confluent monolayers of MDCK cells as described previously (28). The time courses for the viral yields from the supernatant of the medium from the control and the mutant-PKR-transfected cells were similar, suggesting that viral replication was not significantly impaired in these cell lines. Thus, mutant PKR seemed to suppress mainly the Fas-mediated pathway. However, its effect on cell death was partial; therefore, mechanisms other than Fas may also play roles in the cell death as suggested previously (27), and/or the increased basal level of Fas expression in the mutant-PKR-transfected cells contributes to cell death.

It has been reported that influenza virus infection activates the Fas gene, resulting in the apoptosis of cultured cells (27, 32), and it has been suggested that PKR is involved in this process. In this study, we showed that a catalytically inactive mutant PKR suppressed the enhanced Fas expression and cell death caused by virus infection. These findings support the notion that PKR is involved in these processes. About a twofold increase in the promoter activity upon influenza virus infection preceded the increase in the mRNA level. The promoter activity reached a maximum at 2 h postinfection and then decreased to the basal level at 6 h postinfection (32). Therefore, PKR activation in the very early phase of infection may be important for activation of the Fas gene, which is consistent with the data in Fig. 1C. Nucleotide sequence analysis of the 5′ upstream region of the Fas gene has revealed that there are eight repeats of the consensus binding site for the nuclear factor for interleukin 6 expression (NF-IL6) (32). Moreover, the DNA binding activity of NF-IL6 in the nuclear extract from the virus-infected cells increased in a time course similar to that of activation of the promoter, whereas the amount of NF-IL6 was constant (32). NF-IL6 is phosphorylated before activation, and several protein kinases reportedly phosphorylate it (22, 29, 30, 33). Since PKR activates NF-κB by phosphorylating IκB (12), we speculated that PKR also phosphorylates NF-IL6 to activate the Fas gene. This notion is currently under investigation.

The mechanism of the antiviral and antiproliferative actions of PKR remains to be clarified. It seems likely that a loss of translation initiation by phosphorylation of eIF-2α by PKR suppresses cell growth. This is in line with the previous observation that an overexpression of the initiation factor eIF-4E, which enhances translation, induces tumorigenicity (2, 13). However, there is some evidence against this notion, namely, that eIF-2α is phosphorylated in mutant-PKR-transfected cells (18, 19). Therefore, PKR may play different roles in the mechanisms mediating eIF-2α phosphorylation and growth suppression. We suggest that PKR inhibits uncontrolled cell growth, at least in part, through activating a Fas-mediated pathway.

We are grateful to Ara Hovanessian for providing the anti-PKR MAb and PKR cDNA. We also thank Katsuhisa Nakajima and Eri Nobusawa for helpful discussions and support.

This work was supported by the research project program of the Institute for Developmental Research, Aichi Human Service Center, in 1995 and by the Ishida Foundation.

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