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Identification of a Plant-Encoded Analog of PKR, the Mammalian Double-Stranded RNA-Dependent Protein Kinase[1]

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Plant virus or viroid infection stimulates the phosphorylation of a plant-encoded protein of M_r 68,000 to 70,000 (now termed pPKR) that is associated with double-stranded RNA-stimulated protein kinase activity. Using various biochemical and immunological comparisons, we have demonstrated that this plant protein is an analog of the mammalian PKR enzymes. pPKR is both cytosolic and ribosome associated, similar to mammalian PKR, and appears to be capable of phosphorylating exogenous histones. Monoclonal antiserum to the human PKR as well as antiserum to a conserved double-stranded RNA-binding domain present on mammalian PKR demonstrated cross-reactivity with pPKR. Likewise, polyclonal antiserum to the pPKR detected the mouse and human PKR in western blot analysis. Northern blot analysis of a mammalian PKR cDNA detected a specific 2.5-kb transcript present in plant poly(A)$^+$ RNA.

The significance of plant protein phosphorylation in signal transduction, metabolic regulation, and gene expression is now firmly established (Trewavas and Gilroy, 1991; Allen, 1992; Klimczak et al., 1992; Martin et al., 1993) despite inherent difficulties in studying phosphorylation in plant homogenates (Harter et al., 1994). However, structure-function relationships between animal and plant kinases remain to be clarified. Catalytic domains appear to be conserved between plant and animal kinases, although regulatory regions are significantly more diverse (Lawton et al., 1989) and few specific activators of plant kinases, with the exception of Ca^{2+}, have been identified. Comparison with putative analogs in animal cells is one approach to study the regulatory significance of protein phosphorylation in plant cells.

Phosphorylation-dephosphorylation provides an efficient mechanism to regulate host-pathogen interactions. Recently, the resistance gene conditioning specific resistance in tomato to the bacterial pathogen *Pseudomonas syringae* pv *tomato* was cloned and determined to encode a protein kinase (Martin et al., 1993). Plant virus or viroid infection of susceptible host tissue induces the phosphorylation of an M_r 68,000 to 70,000 protein (Crum et al., 1988; Hiddinga et al., 1988). In vitro phosphorylation of this protein is regulated by poly(rI)·poly(rC) or virus dsRNA isolated from infected tissues but not rRNA, DNA, or RNA-DNA hybrids (Roth and He, 1994). However, viroid ssRNA transcripts stimulate the phosphorylation of this plant-encoded protein (J.O. Langland and D.A. Roth, unpublished data). In vivo and in vitro phosphorylation in RNA virus-infected protoplasts also is correlated with early events in viral replication, suggesting a role in pathogenesis (Hu and Roth, 1991). The in vivo phosphorylation of this protein in healthy protoplasts suggests a role in uninfected plants (Hu and Roth, 1991).

The biochemical characteristics of the plant M_r 68,000 to 70,000 protein are similar to those of the vertebrate IFN-induced, dsRNA-dependent protein kinase, PKR (Hovanessian, 1989). Relative to the mPKR, the plant phosphoprotein is present at greater endogenous levels in healthy tissue, although expression of the plant enzyme is induced by virus infection (J.O. Langland and D.A. Roth, unpublished data). Furthermore, the increased level of the M_r 68,000 to 70,000 protein phosphorylation is associated with the susceptible, disease-producing reaction rather than an antiviral response observed with IFN-induced PKR phosphorylation. Characterization of this plant phosphoprotein will contribute to understanding the functional significance of these apparent differences.

In the presence of dsRNA, mPKR autophosphorylates an M_r 68,000 (mouse) or 72,000 (human) protein subunit of PKR, P_1. Autophosphorylation appears to be necessary for PKR activation. Once activated, PKR phosphorylates exogenous substrates, including histone proteins and eIF-2 (Berry et al., 1985; Pestka et al., 1987; Jacobs and Imani, 1988). Phosphorylation of eIF-2 on its α subunit can lead to inhibition of protein synthesis by preventing the exchange of GDP for GTP on the eIF-2 complex, thereby blocking formation of the ternary complex between eIF-2, Met-tRNA, and GTP (Pain, 1986). Through this mechanism, active PKR is likely responsible for the IFN-induced inhibition of replication of several viruses (Sam-

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Abbreviations: ds, double-stranded; eIF-2, eukaryotic protein synthesis initiation factor 2; IFN, interferon; mPKR, mammalian PKR; pPKR, plant PKR; rA, adenosine; rC, cytosine; rI, inosine; S-100, supernatant of the 100,000*g* centrifugation; ss, single-stranded; TMV, tobacco mosaic virus.

uel, 1991). To date, three eIF-2α protein kinases, PKR, the heme-regulated kinase (HCI), and the yeast-encoded GCN2 kinase, have been identified (Samuel, 1993).

It is increasingly clear that PKR is a versatile protein kinase involved in the regulation of diverse cellular processes. Recently, PKR has also been suggested to have a role in the regulation of gene expression, antiproliferative activity, and apoptosis (Clemens, 1992; Koromilas et al., 1992; Lee and Esteban, 1994). Expression and activation of human PKR in yeast results in growth inhibition of transformed cells (Chong et al., 1992). PKR also phosphorylates I-κB, the inhibitor of the transcription factor NF-κB (Clemens, 1992; Kumar et al., 1994). Phosphorylation leads to a dissociation of I-κB from NF-κB and subsequent activation of a number of genes stimulated by NF-κB.

Our initial studies indicating a relationship between the plant M_r 68,000 to 70,000 protein and mPKR were done using crude soluble cytoplasmic extracts and a relatively uncharacterized polyclonal anti-human PKR serum (Crum et al., 1988; Hiddinga et al., 1988). Further purification and characterization of the plant protein has been difficult because of its extreme lability and low abundance. Here we report the further characterization of this plant-encoded phosphoprotein and show that it is a dsRNA-dependent protein kinase (now termed pPKR). The biochemical and enzymatic properties of pPKR are very similar to those observed with mPKR, including dsRNA-stimulated autophosphorylation, specific dsRNA-binding activity, subcellular localization on ribosomes and in the cytosol, and phosphorylation of exogenous histones. Antiserum against the mPKR (monoclonal or domain specific) cross-reacted with the plant-encoded protein and antiserum to the plant enzyme recognized the mPKR. It is significant that northern blot analysis demonstrated that a cDNA probe to the mPKR recognizes a single poly(A)$^+$ RNA from plant cells, which is approximately the same size as the PKR-RNA from mouse and human cells. Taken together, these data suggest the presence of a plant-encoded analog to the mPKR.

MATERIALS AND METHODS

Materials

Monoclonal antiserum to the human PKR has previously been characterized (Laurent et al., 1985) and was kindly provided by A. Hovanassian (Institut Pasteur, Paris, France). Rabbit polyclonal antiserum was generated against a synthetic peptide containing the C-terminal 69-amino acids of the rotavirus group C NSP3 protein that encodes a conserved dsRNA-binding domain (St. Johnston et al., 1992; Langland et al., 1994) and against HPLC-purified pPKR (D.A. Roth, J.O. Langland, and L.A. Langland, unpublished data). Soluble poly(rI)·poly(rC) and poly(rA) were from Sigma. Poly(rI)·poly(rC) agarose was prepared according to the method of Langland et al. (1995). Histone (type IIA) from calf thymus was from Sigma. All materials and chemicals except where indicated were from Sigma.

Cell Growth and Extract Preparation

Cell growth and extract preparation from mouse L cells and human HeLa cells were performed as previously described (Watson et al., 1991; Langland and Jacobs, 1992). Tobacco (*Nicotiana tabacum* L. cv Samsun nn) was grown, mock inoculated, or infected with TMV as previously described (Crum et al., 1988). Raw wheat germ was purchased locally. Barley (*Hordeum vulgare* L. cv Steptoe) was grown in the dark for 7 d at 25°C. Barley grown under ambient light conditions was not used because of inconsistencies in the preparation of the ribosomal pellet.

Barley leaves were homogenized in 50 mM Tris-Cl, pH 7.5, 5 mM EDTA, 5 mM DTT, 100 mM KCl, 1 unit/mL aprotinin, and 2 mM PMSF. Ribosomal salt wash and cytosolic fractions were prepared (Langland and Jacobs, 1992) with the following modifications: homogenized tissue was filtered through four layers of cheesecloth, followed by centrifugation at 40,000g for 30 min. The supernatant fraction was used for preparation of S-100 and ribosomal pellet fractions. The ribosome pellet was resuspended in homogenization buffer containing 1.5 M KCl, followed by recentrifugation. S-100 and ribosomal salt wash fractions were dialyzed overnight at 4°C in 20 mM Tris-Cl, pH 7.5, 10% (v/v) glycerol, 100 mM KCl, 5 mM $MgCl_2$, 5 mM $MnSO_4$, 5 mM DTT, 2 mM PMSF, and 1 unit/mL aprotinin.

Kinase Assays

Kinase assays performed using barley leaf extracts were done under the following conditions (Crum et al., 1988; Hiddinga et al., 1988): 20 mM Tris-Cl, pH 7.5, 100 mM KCl, 5 mM $MgCl_2$, 5 mM $MnSO_4$, 5 mM DTT, 2 mM PMSF, 1 unit/mL aprotinin, and 1 to 5 μM [γ-^{32}P]ATP (100 Ci/mmol). Incubation was done at 25°C for 5 min. Kinase assays on mouse L-cell and human HeLa-cell extracts were performed as previously described using 100 μM [γ-^{32}P]ATP (1 Ci/mmol) at 30°C for 5 min (Langland and Jacobs, 1992).

Poly(rI)·Poly(rC)-Agarose-Binding Assays

Binding assays were performed as previously described (Langland and Jacobs, 1992). For assays using barley fractions, extracts were first incubated for 5 min with 10 mg/mL poly(rA) before incubation with the dsRNA-agarose resin. For competition assays, soluble nucleic acids were incubated with the extract for 5 min at 4°C (at the indicated concentrations) prior to incubation with the dsRNA-agarose.

Western Blot Analysis

Proteins were separated by SDS-PAGE and transferred to nitrocellulose, and nonspecific binding sites were blocked with Blotto (Chu et al., 1989). The nitrocellulose was incubated with the primary antibody and secondary goat anti-rabbit IgG alkaline phosphatase conjugate and developed with 3-hydroxy-2-naphthoic acid anilide phos-

phate and 4-benzoylamino-2,5-diethoxybenzenediazonium chloride hemi[zinc chloride] salt.

Immunoprecipitation

For radioimmune precipitation, extracts were bound to dsRNA-agarose, subjected to in vitro phosphorylation with [γ-^{32}P]ATP, and eluted in 1% (w/v) SDS with boiling. The supernatant fraction was diluted to 0.1% (w/v) SDS, followed by the addition of 5 μL of antiserum and incubation for 1 h on ice. *Staphylococcus aureus* cells (formalin-fixed, heat-killed, Cowan I strain) were added and incubation continued for 1 h. The immunocomplex was pelleted and washed three times in radiolabeled immunoprecipitation antigen buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1% [v/v] Nonidet P-40, 0.5% [v/v] sodium deoxycholate, 0.1% [w/v] SDS) and once in 0.1 M Tris-Cl, pH 7.4, according to the method of Springer (1990). Bound proteins were eluted by addition of SDS-PAGE lysis buffer and incubation for 5 min in a boiling water bath prior to SDS-PAGE and autoradiography (Crum et al., 1988).

For immunoprecipitation-clearing experiments maximal clearing of PKR from extracts was achieved by incubating ribosomal salt wash fractions (0.5 mL) overnight at 4°C with anti-PKR sera (a mixture of a 1:10 dilution of anti-dsRNA-binding domain serum, 1:200 dilution of a monoclonal human anti-PKR serum, and a 1:10 dilution of anti-pPKR serum). Separate fractions were similarly incubated with a 1:10 dilution of preimmune serum. *S. aureus* cells were then added and incubation continued for another 1 h at 4°C. The bacterial cells were pelleted and the supernatant fluid was used in standard poly(rI)·poly(rC)-agarose-binding assays.

Northern Blot Analysis

Leaf tissue (5 g) was harvested, frozen in liquid nitrogen, and ground to a fine powder. The tissue was immediately added to 7.5 mL of NTES (100 mM NaCl, 10 mM Tris, pH 7.5, 1 mM EDTA, 1% [w/v] SDS) and 5 mL of phenol:chloroform:isoamyl alcohol (25:24:1, v/v) and vortexed for 30 s. The extract was centrifuged at 1000*g* for 5 min and the supernatant fraction was recentrifuged at 8000*g* for 10 min. Nucleic acid was precipitated with ethanol and resuspended in 2.5 mL of H_2O. Total RNA was precipitated by the addition of 2.5 mL of 4 M LiCl and overnight incubation at 4°C. The solution was centrifuged at 10,000*g* for 20 min and the pellet was resuspended in H_2O, followed by another precipitation with ethanol. Poly(A)$^+$ RNA was purified from the resulting total RNA using an oligo(dT) column matrix (Oligotex-dT kit; Qiagen, Inc., Chatsworth, CA). Poly(A)$^+$ mRNA (500 ng) was electrophoresed on a 1% (w/v) agarose-formaldehyde gel, transferred to nitrocellulose using a pressure blotter (Posiblot, Stratagene) and fixed by UV cross-linking. The membrane was pretreated with RNA PT buffer (5× SET [20× SET = 3 M NaCl, 0.6 M Tris-Cl, pH 8.0, 40 mM EDTA], 10× Denhardt's solution, 50 mM phosphate buffer, pH 7.4) for 2 h at 42°C (Springer, 1990). The full-length mPKR gene (Meurs et al., 1990) and the mouse PKR gene (Baier et al., 1993) were ^{32}P labeled by primer extension using the Promega Prime-a-Gene Labeling System. After prehybridization, denatured probe was added in hybridization solution (20% [v/v] formamide, 5× SET, 1× Denhardt's solution, 20 mM phosphate buffer, pH 7.4, 1% [w/v] SDS, 100 μg/mL sheared/denatured salmon sperm DNA) and incubated overnight at 42°C. The filter was subsequently washed twice each in 4× SSC (20× SSC = 3 M NaCl, 0.3 M sodium citrate, pH 7.5) with 0.2% (w/v) SDS, 1× SSC with 0.2% (w/v) SDS, and 0.1× SSC with 0.2% (w/v) SDS and subsequently visualized by autoradiography.

RESULTS

dsRNA-Dependent Autophosphorylation and Binding Properties of pPKR

Activation of the mPKR is dependent on the presence of dsRNA (Hovanessian, 1989). As shown in Figure 1B, autophosphorylation of an M_r 68,000 to 70,000 protein (mouse PKR) in ribosomal salt wash extracts from mouse L cells occurred in the presence of 1 μg/mL dsRNA. This autophosphorylation event leads to the activation of mPKR (Samuel, 1993). The radiolabeled minor band at M_r 80,000 in Figure 1B has been observed previously from mouse cell extracts, but the identity is not known (B.L. Jacobs, unpublished data). When similar assays were performed using barley leaf extracts, dsRNA-dependent phosphorylation of an M_r 70,000 protein (pPKR) was observed (Fig. 1A). Much higher concentrations of dsRNA were required for pPKR phosphorylation (5–25 μg/mL) than for mPKR; however, this may be due to the presence of nuclease activity in the crude barley extract.

As previously reported (Watson et al., 1991), both human and mouse PKR bind specifically to dsRNA-agarose and can subsequently be autophosphorylated in the presence of ATP (Fig. 2, lanes A and B). Since pPKR phosphorylation was dependent on the presence of dsRNA, pPKR binding

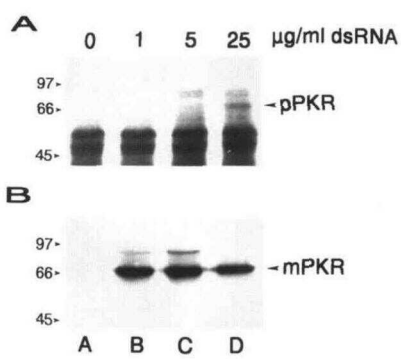

Figure 1. dsRNA-dependent phosphorylation of a barley M_r 68,000 to 70,000 polypeptide (pPKR). Barley (A) or mouse L-cell (B) ribosomal salt wash extracts were prepared and incubated in the presence of [γ-^{32}P]ATP with increasing concentrations of soluble poly(rI)·poly(rC): lane A, 0 μg/mL; lane B, 1 μg/mL; lane C, 5 μg/mL; lane D, 25 μg/mL. The figure represents the autoradiograph of radiolabeled proteins separated by SDS-PAGE. Molecular mass markers (kD) are shown to the left.

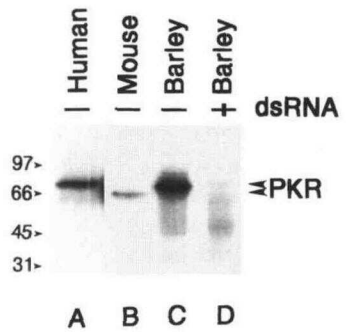

Figure 2. dsRNA-agarose binding and phosphorylation of the barley leaf pPKR. Human HeLa-cell (A), mouse L-cell (B), or barley (C and D) ribosomal salt wash extracts were bound to poly(rI)·poly(rC)-agarose and thoroughly washed and the matrix was subjected to in vitro phosphorylation in the presence of [γ-^{32}P]ATP. In D, the barley leaf extract was incubated with a 5-fold excess of soluble dsRNA prior to incubation with the dsRNA-agarose. Bound proteins were eluted, separated by SDS-PAGE, and visualized by autoradiography. Molecular mass markers (kD) are shown to the left.

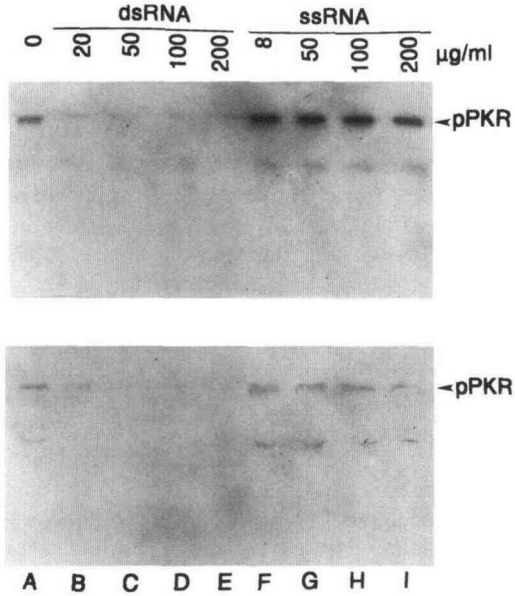

Figure 3. Specific dsRNA binding of barley leaf pPKR. The barley ribosomal salt wash fraction was incubated with increasing concentrations of soluble poly(rI)·poly(rC) (B–E) or soluble poly(rA) (F–I) prior to incubation with poly(rI)·poly(rC)-agarose. The contents of lane A were not preincubated with free nucleic acid. Bound proteins were subjected to in vitro phosphorylation with [γ-^{32}P]ATP, eluted, separated by SDS-PAGE, and transferred to nitrocellulose. Proteins were visualized by autoradiography (top) or western blotting using antiserum developed against the barley pPKR protein (bottom).

to a dsRNA-linked affinity resin was determined. Initial attempts measuring pPKR binding to dsRNA-agarose were inconsistent. We determined that preincubation of the plant extracts with 10 µg/mL ssRNA prior to incubation with the dsRNA-agarose resin was necessary for binding and phosphorylation. This is likely due to the removal of nonspecific RNA-binding proteins that would otherwise inhibit pPKR binding and phosphorylation in this assay. Following ssRNA preincubation with barley leaf extracts, specific binding of pPKR protein to the dsRNA resin and subsequent autophosphorylation in the presence of ATP were observed (Fig. 2, lane C). Binding of the pPKR to the dsRNA-agarose could be prevented by the addition of excess soluble dsRNA (Fig. 2, lane D). As shown in Figure 2, the human and mouse PKRs have M_rs of 72,000 and 68,000, respectively. The pPKR appears to have an M_r of approximately 70,000 by SDS-PAGE.

Competition assays were performed to determine the specificity of dsRNA binding by pPKR. Barley leaf extracts were preincubated with 10 mg/mL ssRNA. Following this preincubation, increasing concentrations of soluble dsRNA or soluble ssRNA as indicated were added, and the extracts were incubated with dsRNA-agarose. pPKR binding and phosphorylation on the dsRNA-agarose was assayed after thorough washing of the resin. As shown in Figure 3, in the presence of soluble dsRNA subsequent pPKR phosphorylation levels decreased (top, cf. lane A with lanes B–E); however, no decrease in pPKR phosphorylation levels were observed in the presence of increasing amounts of soluble ssRNA (top, cf. lane A with lanes F–I). To confirm that the decrease in pPKR phosphorylation levels was due to a decrease in pPKR protein levels, antiserum specific to pPKR was used. As shown in the western blot in Figure 3 (bottom), pPKR protein levels decreased in a manner comparable to phosphorylation levels in the presence of soluble dsRNA (lanes B–E). Little or no decrease in pPKR protein levels was observed upon incubation when ssRNA was added prior to dsRNA-agarose-binding assays (lanes F–I).

Taken together, these data indicate that soluble dsRNA but not ssRNA specifically competes for pPKR binding to dsRNA-agarose, causing subsequent inhibition of autophosphorylation.

Subcellular Distribution of pPKR

At the subcellular level, the mPKR is present both free in the cytosol and in association with ribosomes (Fig. 4, lanes

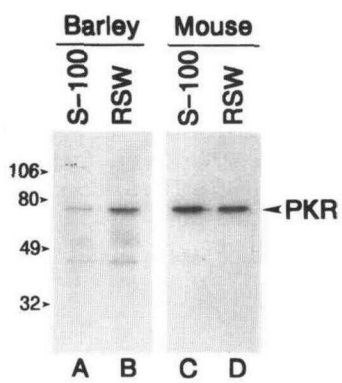

Figure 4. Subcellular distribution of barley leaf pPKR. Cytosolic (A and C) and ribosomal salt wash (RSW; B and D) fractions from barley (A and B) and mouse L cells (C and D) were prepared as described in the text. Extracts were incubated with poly(rI)·poly(rC)-agarose, washed, and phosphorylated with [γ-^{32}P]ATP. Bound proteins were eluted, separated by SDS-PAGE, and visualized by autoradiography. Molecular mass markers (kD) are shown to the left.

C and D; Langland and Jacobs, 1992). Presumably, this is important in the functional and physiological roles of the mPKR. Similar subcellular fractions were prepared from barley leaf tissue. When barley cytosolic (S-100) and ribosomal salt wash fractions were assayed for pPKR activity, a similar M_r 70,000 phosphoprotein was detected in both fractions (Fig. 4, lanes A and B, respectively). Western blot analysis using antiserum to pPKR and to the specific dsRNA-binding domain peptide also indicated the presence of the M_r 70,000 radiolabeled pPKR protein in both subcellular fractions (data not shown).

dsRNA-Dependent Histone Phosphorylation

The mPKR is known to phosphorylate several substrates, including histone proteins (Jacobs and Imani, 1988). Histone phosphorylation requires activation of mPKR and, therefore, coincides with mPKR autophosphorylation. dsRNA-dependent phosphorylation of histone proteins by mPKR is shown in Figure 5B. Assays were performed to determine whether similar dsRNA-dependent phosphorylation of histone proteins could be observed in barley ribosomal salt wash extracts. Extracts of barley leaf tissue contained histone kinase activity even in the absence of dsRNA (Fig. 5A). However, addition of dsRNA led to an increase in histone phosphorylation, which was most evident with H1 histone (M_r 38,000). Plant PKR autophosphorylation is not visible on the short film exposure shown in this figure but is visible upon longer exposure (data not shown). Maximal pPKR autophosphorylation was detected at 30 μg/mL dsRNA, a concentration similar to that which led to maximal histone phosphorylation. When cytosolic and ribosomal salt wash extracts from barley leaves were purified by dsRNA-agarose chromatography, the bound protein fraction was capable of catalyzing histone phosphorylation (data not shown).

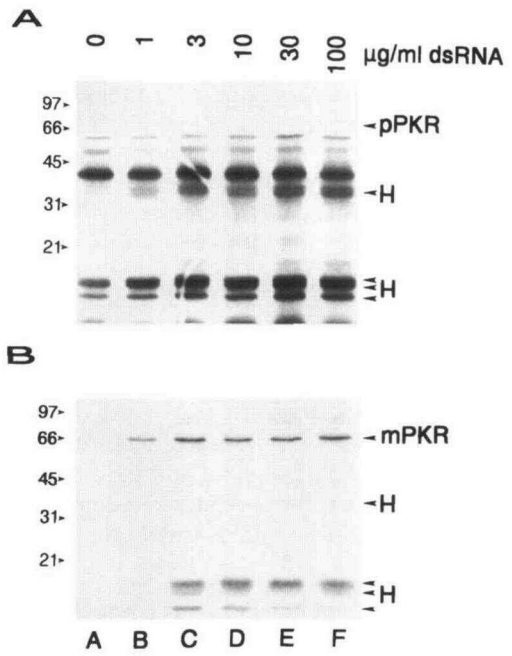

Figure 5. dsRNA-dependent phosphorylation of exogenous histone proteins by barley leaf extracts. Barley (A) or mouse L-cell (B) ribosomal salt wash extracts were incubated in the presence of [γ-^{32}P]ATP, 300 μg/mL histone proteins (H; type IIA from calf thymus), and increasing concentrations of soluble poly(rI)·poly(rC): lane A, 0 μg/mL; lane B, 1 μg/mL; lane C, 3 μg/mL; lane D, 10 μg/mL; lane E, 30 μg/mL; lane F, 100 μg/mL. Proteins were separated by SDS-PAGE and visualized by autoradiography. Molecular mass markers (kD) are shown to the left.

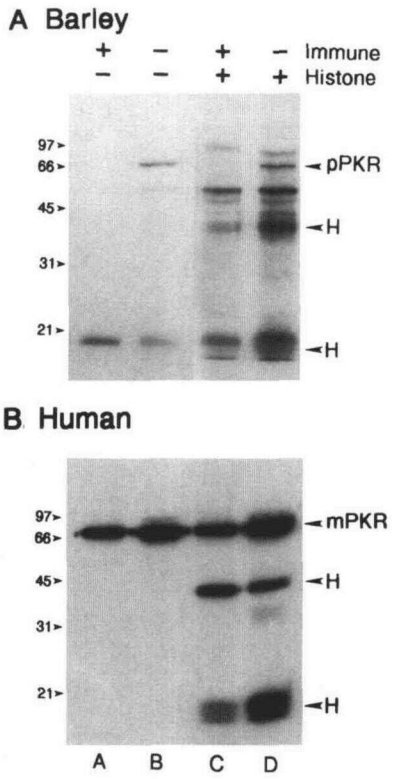

Figure 6. Decreased histone phosphorylation by PKR in immunocleared extracts. Extracts containing pPKR from the barley ribosomal salt wash (A) or mPKR partially purified from HeLa cells according to the method of Langland and Jacobs (1992) (B) were incubated with preimmune (lanes B and D) or a mixture of anti-PKR sera (lanes A and C). The antiserum was precipitated and the supernatant fraction assayed for kinase activity on dsRNA-agarose in the presence (lanes C and D) or absence (lanes A and B) of histone proteins (H; type IIA from calf thymus). Proteins were separated by SDS-PAGE and visualized by autoradiography.

To further demonstrate pPKR involvement in histone phosphorylation, immunoprecipitation clearing assays were performed. In this assay, anti-PKR or preimmune serum was incubated with extracts, followed by precipitation of the antiserum with *S. aureus* cells. The supernatant fraction was used in a poly(rI)·poly(rC)-agarose-binding assay to monitor PKR autophosphorylation and histone phosphorylation. As shown in Figure 6, levels of pPKR autophosphorylation were reduced upon clearing with immune but not with preimmune sera (cf. lanes A and B of Fig. 6A). When these fractions were assayed for the ability to phosphorylate exogenous histones, a reduction in the level of histone phosphorylation was also observed (Fig. 6A, lanes C and D). Similar results were obtained when

human extracts were assayed. Immune sera reduced the level of mPKR autophosphorylation to some degree and reduced the phosphorylation of exogenous histone proteins, except histone H1 (Fig. 6B, lanes A–D). Such differential histone phosphorylation has been previously observed with the mPKR (Jacobs and Imani, 1988).

Immunocharacterization of pPKR

A highly specific, monoclonal antiserum to the human PKR (Laurent et al., 1985) and a rabbit polyclonal antiserum to the 69-amino acid dsRNA-binding motif of the rotavirus NSP3 protein (Langland et al., 1994) were used to characterize antigenic similarities between the mPKR and pPKR proteins. Immunoprecipitation analysis demonstrated that both the anti-dsRNA-binding motif serum and the monoclonal anti-human PKR serum recognized dsRNA-agarose-purified human PKR (Fig. 7, lanes B and D, respectively). Similar immunoprecipitation results were obtained with the dsRNA-agarose-purified barley pPKR (Fig. 7, lanes A and C). A co-precipitated protein of M_r approximately 48,000 was also detected in both the human and barley extracts. This protein has previously been reported in the human system as a degradation product of the full-length PKR protein (Galabru and Hovanessian, 1985).

Western blot analysis also demonstrated immunological similarity between mPKR and pPKR. An M_r 70,000 band

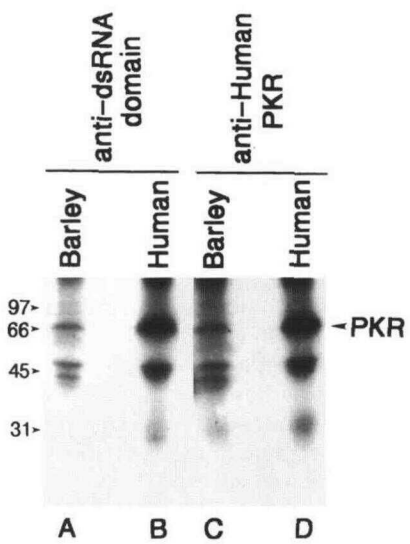

Figure 7. Human PKR monoclonal antiserum and antiserum to a conserved dsRNA-binding motif immunoprecipitate both the human PKR protein and barley pPKR. Ribosomal salt wash extracts from barley (A and C) and human HeLa cells (B and D) were bound to poly(rI)·poly(rC)-agarose, incubated with [γ-^{32}P]ATP, and eluted in SDS-containing buffer. Eluted proteins were then incubated with antiserum to either a conserved dsRNA-binding motif previously identified in several dsRNA-binding proteins (St. Johnston et al., 1992; Langland et al., 1994) (A and B) or monoclonal antiserum against human PKR (Laurent et al., 1985) (C and D). Immunocomplexes were then precipitated using *S. aureus* cells, and the precipitated proteins were analyzed by SDS-PAGE and autoradiography. Molecular mass markers (kD) are shown to the left.

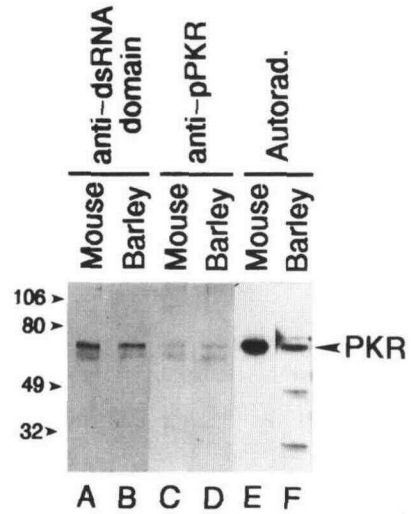

Figure 8. Cross-immunoreactivity between the mPKR and the barley pPKR by western blot analysis. Barley (B, D, and F) or mouse L-cell (A, C, and E) ribosomal salt wash extracts were bound to poly(rI)·poly(rC)-agarose and incubated in the presence of [γ-^{32}P]ATP. Bound proteins were eluted, separated by SDS-PAGE, transferred to nitrocellulose, and analyzed by western blot analysis using antiserum to the conserved dsRNA-binding motif (A and B) and to HPLC-purified pPKR (C and D). E and F represent the autoradiograph of the western blot. Molecular mass markers (kD) are shown to the left.

was recognized by antiserum to the conserved dsRNA-binding domain and co-migrated with the phosphorylated protein (Fig. 8, cf. lanes A and B with lanes E and F). This band was present on western blots using mouse and barley ribosomal salt wash extracts in which protein had first been purified on dsRNA-agarose (Fig. 8, lanes A and B, respectively). Furthermore, polyclonal antiserum to HPLC-purified pPKR (J.O. Langland, L.A. Langland, and D.A. Roth, unpublished data) was able to detect a dsRNA-agarose-purified protein present in both mouse and barley extracts (Fig. 8, lanes C and D, respectively). Doublet bands were detected on the western blot of barley and mouse ribosomal salt wash fractions using either the anti-dsRNA-binding motif serum or the anti-pPKR serum. The upper band co-migrated with the phosphorylated protein, and the lower band is thought to represent either the unphosphorylated form of PKR (Langland and Jacobs, 1992) or a degradation product of the protein. Preimmune sera did not cross-react with pPKR or mPKR (data not shown).

Northern Blot Analysis of Tobacco and Wheat Germ RNA

Radiolabeled probes were created by random-priming reactions using cDNA clones of the mouse (Baier et al., 1993) and human (Meurs et al., 1990) PKRs. Northern blot analysis using these probes revealed primarily a single transcript present in human poly(A)$^+$ RNA (Fig. 9, lane D). A similarly sized 2.5-kb transcript was also detected from poly(A)$^+$ RNA isolated from mock-inoculated and TMV-infected tobacco leaf tissue and from wheat germ using these probes (Fig. 9, lanes A–C, respectively).

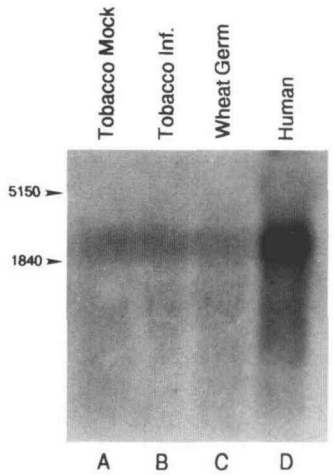

Figure 9. Detection of mPKR-specific transcripts in plants. Poly(A)$^+$ RNA was purified from mock-infected (Mock) and TMV-infected (Inf.) tobacco leaves (lanes A and B, respectively), wheat germ (lane C), and HeLa cells (lane D), separated on a 1% (w/v) agarose-formaldehyde gel, and transferred to nitrocellulose. The nitrocellulose filter was probed with a 50:50 (v/v) mixture of ^{32}P-labeled human and mouse cDNA. Hybridization of the probe was visualized by autoradiography. The migratory positions of rRNAs are indicated in bp.

DISCUSSION

Phosphorylation/dephosphorylation is an important mechanism by which the functional activity of proteins can be regulated. The IFN-induced, dsRNA-dependent protein kinase PKR has been suggested to regulate multiple biological processes, including virus replication (Hovanessian, 1989; Samuel, 1991; Sen and Lengyel, 1992). Previous studies, using nucleotide photoaffinity labeling and immunoprecipitation assays, identified a plant-encoded protein whose phosphorylation is induced in the presence of virus or viroid infection or treatment with dsRNA (Crum et al., 1988; Hiddinga et al., 1988) but not dsDNA or DNA:RNA hybrids (Roth and He, 1994). Here we demonstrate that this protein, now termed pPKR, is analogous to mPKR. Physical characteristics of pPKR are similar to those observed with the mPKR, including dsRNA-binding activity and dsRNA-dependent phosphotransferase activity. Furthermore, three different specific antisera cross-reacted with the mPKR and pPKR. One of the antisera was specific to a conserved dsRNA-binding motif (St. Johnston et al., 1992; Langland et al., 1994), and its recognition of the pPKR suggests the presence of this conserved domain in the pPKR protein. Specific binding of pPKR to dsRNA-agarose further supports this contention. The presence of a specific dsRNA-binding site in the pPKR suggests conservation of a regulatory motif between plant and vertebrate kinases. The catalytic domains of vertebrate and plant kinases show substantial homology; however, conservation of regulatory domains, such as the dsRNA-binding domain, has not been generally observed (Lawton et al., 1989).

The hybridization of cDNAs from mouse and human PKR to a single 2.5-kb poly(A)$^+$ RNA from plant cells provides further evidence establishing the presence of a PKR analog in plant tissue. Based on this study, we are currently screening plant cDNA libraries and have identified multiple positive clones using mouse and human cDNA probes. Sequencing of these clones will directly establish the homology between the mPKR enzymes and pPKR.

The demonstration of a PKR analog in plants has implications in the regulation of viral and viroid pathogenesis as well as in normal cellular function. Although the function of pPKR remains speculative, the enzyme may mediate the host response to viral or viroid infection through the presence of dsRNA in a system analogous to mPKR. It is unknown whether plant eIF-2α can be phosphorylated by pPKR, although our preliminary evidence suggests that human and plant eIF-2α may be phosphorylated in vitro by dsRNA-agarose-purified pPKR. The specificity and nature of this phosphorylation event remains to be studied. Clearly, a shutdown of protein synthesis in plant cells is not observed upon virus infection. However, Fraser and Gerwitz (1980) found that protein synthesis in TMV-infected tissues can be significantly inhibited during virus replication with subsequent recovery to levels comparable to those in uninfected plants following virus accumulation. No change in host mRNA levels was found, suggesting that inhibition was at the level of translation. In the mammalian system, many viruses have evolved regulatory mechanisms to inhibit mPKR activity and thus the antiviral effects associated with mPKR (Kitajewski et al., 1986; Jacobs and Imani, 1988; O'Malley et al., 1989; Watson et al., 1991; Katze, 1992). Thus, it is conceivable that, although pPKR phosphorylation is significantly increased upon TMV infection of a susceptible host genotype (Crum et al., 1988), subsequent antiviral effects may be inhibited.

Alternately, our initial studies suggested that activation of pPKR may represent the triggering event in virus and viroid pathogenesis (Hiddinga et al., 1988; Hu and Roth, 1991). In vivo pPKR phosphorylation in tobacco protoplasts synchronously infected with TMV is correlated with virus replication and synthesis of viral proteins (Hu and Roth, 1991). Viroids replicate and induce disease in plants without encoding any proteins. This implies that the highly structured viroid RNA or replicative intermediates may interact with selected host factors to regulate replicative processes and symptom development. Recently, Diener et al. (1993) found that the mPKR is capable of being activated by viroid strains in a manner directly related to their ability to incite severe disease symptoms. These data, together with the observation that pPKR phosphorylation levels are induced during viroid infection (Hiddinga et al., 1988), support a possible role for pPKR in pathogenesis.

Although a major role of PKR in mammalian cells relates to the IFN-induced antiviral response, it is now apparent that mPKR is multifunctional. The mPKR is believed to be involved in control of gene expression through phosphorylation of I-κB. Phosphoryation induces the dissociation of I-κB from NF-κB and activation of the transcription of a number of genes stimulated by NF-κB (Clemens, 1992; Jimenez-Garcia et al., 1993; Kumar et al., 1994). Recently, mPKR has also been suggested to have apparent tumor-

suppressor activity (Clemens, 1992; Koromilas et al., 1992), possibly through translational and/or transcriptional regulatory mechanisms or phosphorylation of other regulatory substrates, perhaps including histone proteins. The distinctly different subcellular forms of mPKR have been suggested to be involved in these multiple physiological roles of mPKR (Koromilas et al., 1992; Langland and Jacobs, 1992). Since the pPKR has a subcellular distribution similar to that described for the mPKR, comparable physiological activities may be expected. In plant extracts, both ribosome-associated and cytosolic fractions containing pPKR were able to phosphorylate exogenous histones. Furthermore, pPKR has dsRNA-binding activity and could phosphorylate histones in a dsRNA-dependent manner. The dsRNA-dependent phosphorylation of histones paralleled pPKR phosphorylation and immunoclearing of pPKR from extracts resulted in a reduction in the extent of histone phosphorylation. These results suggest that histone proteins may be a substrate for pPKR. The functional significance of histone phosphorylation by the mPKR remains unclear. However, recent evidence suggests the cell-cycle-dependent presence of mPKR within the nucleus (Jimenez-Garcia et al., 1993), thereby possibly giving mPKR access to histone substrates. The involvement of mPKR with apoptosis also suggests the regulatory significance of PKR and a possible role of histone phosphorylation (Lee and Esteban, 1994).

The multifunctional nature of mPKR suggests several possible roles for the analogous pPKR in infected as well as healthy plant cells. The cloning of pPKR will provide the opportunity to explore many of these potential effects.

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