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Mechanism of Interferon Action: Characterization of the Intermolecular Autophosphorylation of PKR, the Interferon-Inducible, RNA-Dependent Protein Kinase

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The interferon-inducible, RNA-dependent protein kinase (PKR) is activated by autophosphorylation, a process mediated by double-stranded RNA. A catalytically deficient, histidine-tagged mutant PKR protein [His-PKR(K296R)] was used as the substrate for characterization of the intermolecular phosphorylation catalyzed by purified wild-type PKR [PKR(Wt)]. The intermolecular autophosphorylation of His-PKR(K296R) by PKR(Wt) was RNA dependent. Excess His-PKR(K296R) substrate inhibited both the auto- and the *trans*-phosphorylation activities of PKR(Wt). Inhibition of PKR(Wt) by His-PKR(K296R) was relieved by higher concentrations of activator double-stranded RNA. Phosphopeptide analysis revealed that the sites of intermolecular autophosphorylation in His-PKR(K296R) were very similar, if not identical, to the sites that were autophosphorylated in PKR(Wt) and suggest a multiple of four major phosphorylation sites per PKR molecule.

The α subunit of eukaryotic protein synthesis initiation factor 2 (eIF-2) is one of the translation factors whose activity is modulated by phosphorylation (10). The interferon-inducible, RNA-dependent protein kinase PKR catalyzes the phosphorylation of eIF-2 (18, 20). Phosphorylation of eIF-2 by PKR results in inhibition of mRNA translation (10, 18). The amount of functional PKR kinase activity in mammalian cells is regulated by interferon at the level of PKR gene transcription (16, 21, 22) and by RNA at the level of activation of PKR kinase activity (4, 6, 13, 17). In the presence of a suitable activator RNA, PKR becomes autophosphorylated mainly on serine and threonine residues, leading to activation of PKR enzymic activity (7, 14, 17).

The mechanism of PKR autophosphorylation and subsequent kinase activation is not well understood. Previous studies have suggested that RNA-dependent autophosphorylation of PKR can occur by either inter- or intramolecular mechanisms (4, 8, 12). Recent studies definitively established that RNA-dependent autophosphorylation of PKR may indeed include intermolecular phosphorylation events, because histidine-tagged PKR(K296R) is a substrate for the wild-type PKR [PKR(Wt)] kinase via an intermolecular *trans*-phosphorylation mechanism (24). The K296R mutation, which substitutes arginine for lysine at position 296 of kinase catalytic subdomain II, destroys kinase activity (11, 23). The engineered N-terminal histidine label adds about 4 kDa to the size of PKR(K296R) and thus allows electrophoretic separation of PKR(Wt) and His-PKR(K296R) (24).

In this communication, we further characterize the intermolecular, RNA-dependent phosphorylation of the mutant His-PKR(K296R) catalyzed by PKR(Wt). The optimal concentration of double-stranded RNA (dsRNA) required to activate PKR(Wt) was dependent on the concentration of the His-PKR(K296R) substrate; furthermore, at limiting dsRNA concentrations, excess His-PKR(K296R) antagonized the autophosphorylation of PKR(Wt). The sites that were *trans*-autophosphorylated in His-PKR(K296R) were compared by phosphopeptide analysis with those that were autophosphorylated in PKR(Wt). Most, if not all, of the sites that were phosphorylated in the His-PKR(K296R) substrate were identical to those autophosphorylated within the PKR(Wt) enzyme.

RNA dependence of phosphorylation of His-PKR(K296R). To further characterize the *trans*-autophosphorylation of His-PKR(K296R) and examine the effect of His-PKR(K296R) on PKR(Wt) autophosphorylation, we carried out mixing reactions at various dsRNA concentrations with purified PKR(Wt) and purified recombinant His-PKR(K296R). As shown in Fig. 1, both the autophosphorylation of PKR(Wt) (Fig. 1A) and the *trans* phosphorylation of His-PKR(K296R) (Fig. 1B) were RNA dependent. Furthermore, addition of increasing amounts of His-PKR(K296R) substrate to the in vitro phosphorylation reaction mixture while holding constant the amount of PKR(Wt) enzyme caused a shift of the activation curve of PKR(Wt) towards higher RNA concentrations (Fig. 1A). In the absence of His-PKR(K296R), maximal activation of PKR(Wt) autophosphorylation was achieved in the presence of 0.1 μg of poly(rI)-poly(rC) activator dsRNA per ml. Addition of a small amount of His-PKR(K296R) (a twofold molar excess) to the reaction mixture did not significantly affect the PKR(Wt) activation curve (Fig. 1A). However, when the reaction mixture contained a 10- or 50-fold molar excess of His-PKR(K296R), maximal autophosphorylation of PKR(Wt) required correspondingly higher concentrations of poly(rI)-poly(rC), 0.5 and 1.0 μg/ml, respectively (Fig. 1A). In a parallel manner, *trans* phosphorylation of His-PKR(K296R) likewise required higher concentrations of dsRNA when the amount of His-PKR(K296R) was increased relative to the amount of PKR(Wt) (Fig. 1B). These results suggest that His-PKR(K296R), which is defective in kinase catalytic activity (11, 23) but not RNA-binding activity (15), sequestered activator RNA, thereby resulting in a shift of the

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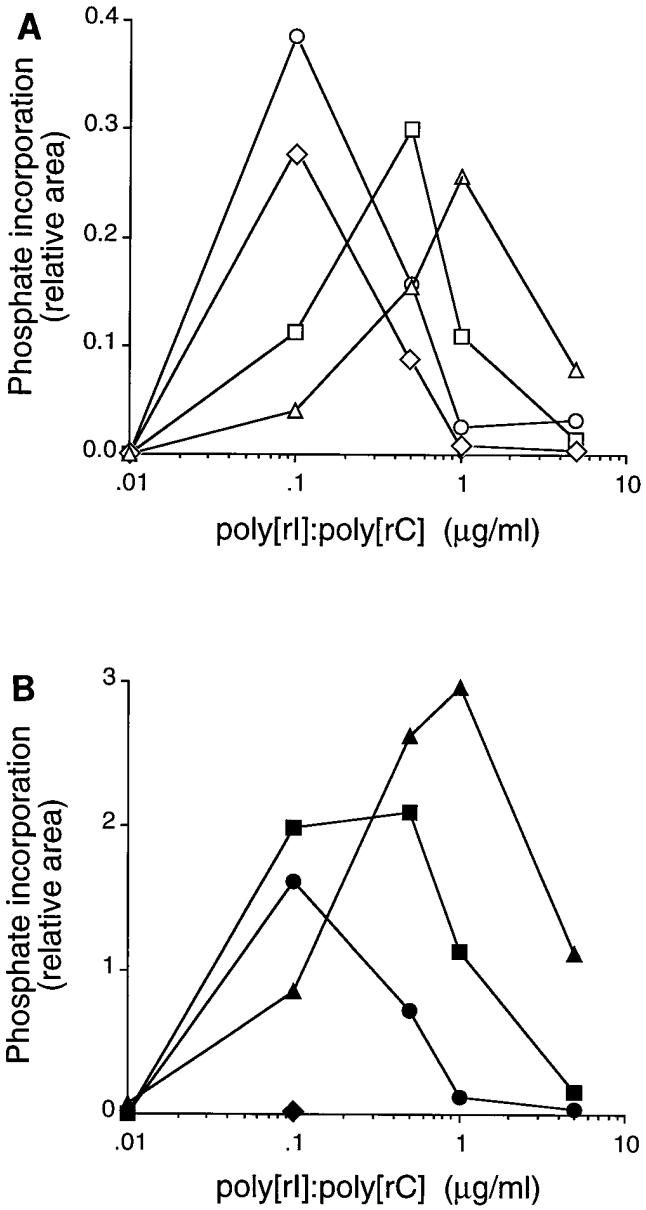

FIG. 1. RNA dependence of phosphorylation of His-PKR(K296R). [γ-^{32}P]ATP-mediated phosphorylation of PKR(Wt) and HIS-PKR(K296R) was carried out as previously described (19, 23). (A) PKR(Wt) autophosphorylation. In vitro phosphorylation reactions contained 0.01, 0.1, 0.5, 1.0, and 5.0 μg of poly(rI)-poly(rC) and PKR(Wt) alone [◇; 0:1 K296R-PKR(Wt) ratio] or PKR(Wt) and a 2-fold (○; 2:1), 10-fold (□; 10:1), or 50-fold (△; 50:1) molar excess of His-PKR(K296R). (B) His-PKR(K296R) *trans* phosphorylation. In vitro phosphorylation reactions contained 0.01, 0.1, 0.5, 1.0, and 5.0 μg of poly(rI)-poly(rC) and His-PKR(K296R) alone [◆; 1:0 K296R-PKR(Wt) ratio] or PKR(Wt) and a 2-fold (●; 2:1), 10-fold (■; 10:1), or 50-fold (▲; 50:1) molar excess of His-PKR(K296R). Reaction mixtures were incubated at 30°C for 2 min, ^{32}P-labeled phosphoproteins were fractionated by SDS–10% PAGE, and the autoradiograms were quantitated by laser densitometry. The PKR(Wt) protein, obtained by MonoQ ion-exchange fast protein liquid chromatography of the ribosomal salt wash prepared from interferon-treated human amnion U cells (19), was kindly provided by S. J. McCormack of our laboratory. For production of the His-PKR(K296R) protein, the cDNA encoding full-length PKR(K296R) (23) was subcloned into pBlueBacHisC (InVitroGen) and a recombinant baculovirus was produced (24). The His-PKR(K296R) protein was purified 45 h after infection of Sf21 cells. The washed cell pellet was resuspended in lysis buffer (20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.5], 0.5 M KCl, 0.65% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 10 μg of aprotinin per ml, 2 μg of leupeptin per ml), and a 100,000 × *g* supernatant fraction was prepared. His-PKR(K296R) was purified from the supernatant fraction by Ni^{2+}-chelating Sepharose column chromatography (fast flow metal-chelating Sepharose; Pharmacia). The 100 mM imidazole eluate containing His-PKR(K296R) was dialyzed against MonoS buffer (20 mM HEPES [pH 7.5], 0.1 mM EDTA, 100 mM KCl, 0.01% Triton X-100, 10% glycerol, 5 mM β-mercaptoethanol, 0.5 mM phenylmethylsulfonyl fluoride) and then further fractionated by MonoS ion-exchange fast protein liquid chromatography with a linear salt gradient of 100 to 500 mM KCl in MonoS buffer. MonoS fractions were dialyzed against MonoQ buffer (20 mM HEPES [pH 7.5], 50 mM KCl, 2.5 mM $MgCl_2$, 2.5 mM $MnCl_2$, 0.01% Triton X-100, 10% glycerol, 5 mM β-mercaptoethanol, 0.5 mM phenylmethylsulfonyl fluoride).

PKR(Wt) enzyme activation curve to a higher RNA concentration as the concentration of His-PKR(K296R) was increased.

Kinetics of phosphorylation of His-PKR(K296R). To verify that the phosphorylation of PKR was near saturation prior to phosphopeptide analyses of His-PKR(K296R), the kinetics of the in vitro phosphorylation reaction were examined. In the presence of equimolar amounts of PKR(Wt) and His-PKR(K296R), phosphorylation of His-PKR(K296R) was complete after incubation for 2 min; when the His-PKR(K296R) substrate was present at a twofold molar excess, maximal intermolecular autophosphorylation of His-PKR(K296R) was reached after 10 min (data not shown). As shown in Fig. 2, when the reaction contained either a 10-fold (Fig. 2A, lanes 1 to 4) or a 50-fold (Fig. 2A, lanes 5 to 8) molar excess of His-PKR(K296R), the kinetics of phosphate incorporation into His-PKR(K296R) were similar; about 30 min was required to obtain maximal phosphorylation of His-PKR(K296R) (Fig. 2B). The phosphorylation kinetics of PKR(Wt) were similar at both concentrations of His-PKR(K296R), although saturation of PKR(Wt) phosphorylation appeared to be reached much more rapidly than that of His-PKR(K296R). These results suggest that when the in vitro phosphorylation reaction was carried out with the poly(rI)-poly(rC) concentration optimized for the amount of added His-PKR(K296R) substrate, the intermolecular autophosphorylation of His-PKR(K296R) catalyzed by the PKR (Wt) enzyme was saturated at a substrate-enzyme ratio of about 10:1.

Phosphopeptide analyses of His-PKR(K296R) and PKR(Wt). To compare the sites of intermolecular phosphorylation of His-PKR(K296R) with the sites of autophosphorylation of PKR(Wt), the profiles of chymotryptic phosphopeptides were compared. His-PKR(K296R) was *trans* phosphorylated by PKR(Wt) in vitro, separated from PKR(Wt) by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 2A), transferred to nitrocellulose, and digested with chymotrypsin; the peptides were then fractionated by high-pressure liquid chromatography (HPLC).

Three major radioactive peaks (Ψ1, Ψ2, and Ψ3) and three or four minor peaks were reproducibly observed in the chymotryptic phosphopeptide profile of *trans* autophosphorylated His-PKR(K296R) (Fig. 3A). The retention times of the three major peaks were 32.0 (Ψ1), 38.5 (Ψ2), and 42.0 (Ψ3) min, respectively. Peaks Ψ2 and Ψ3 contained similar amounts of radioactivity; peak Ψ1 contained about twice as much radioactivity as peaks Ψ2 and Ψ3. If peaks Ψ2 and Ψ3 correspond to phosphopeptides that contain a single phosphoamino acid residue, then peak Ψ1 would likely represent two phosphoamino acid residues. Thus, it appears that His-PKR(K296R) contains at least four major phosphorylation sites. It is unclear whether the minor peaks eluting at 2.5, 25.5, 37.0, 74.0, and 79.5 min represent additional minor phosphorylation sites that were utilized less efficiently or whether they resulted from incomplete or nonspecific protease digestion of His-PKR(K296R).

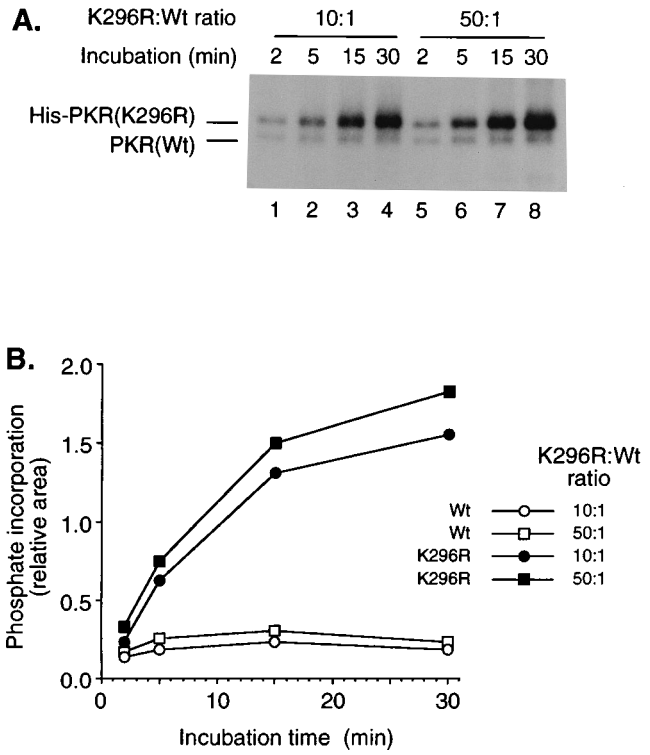

FIG. 2. Kinetics of phosphorylation of His-PKR(K296R). (A) Phosphorylation of PKR(Wt) and His-PKR(K296R). In vitro phosphorylation reactions contained PKR(Wt) and 0.1 μg of poly(rI)-poly(rC) and either a 10- or 50-fold molar excess of His-PKR(K296R). Reaction mixtures were incubated at 30°C for 2, 5, 15, or 30 min, and proteins were fractionated by SDS–10% PAGE. (B) Quantitation of the autoradiogram shown in panel A by laser densitometry. Symbols: ○ and □, incorporation of phosphate into PKR(Wt); ● and ■, incorporation of phosphate into His-PKR(K296R).

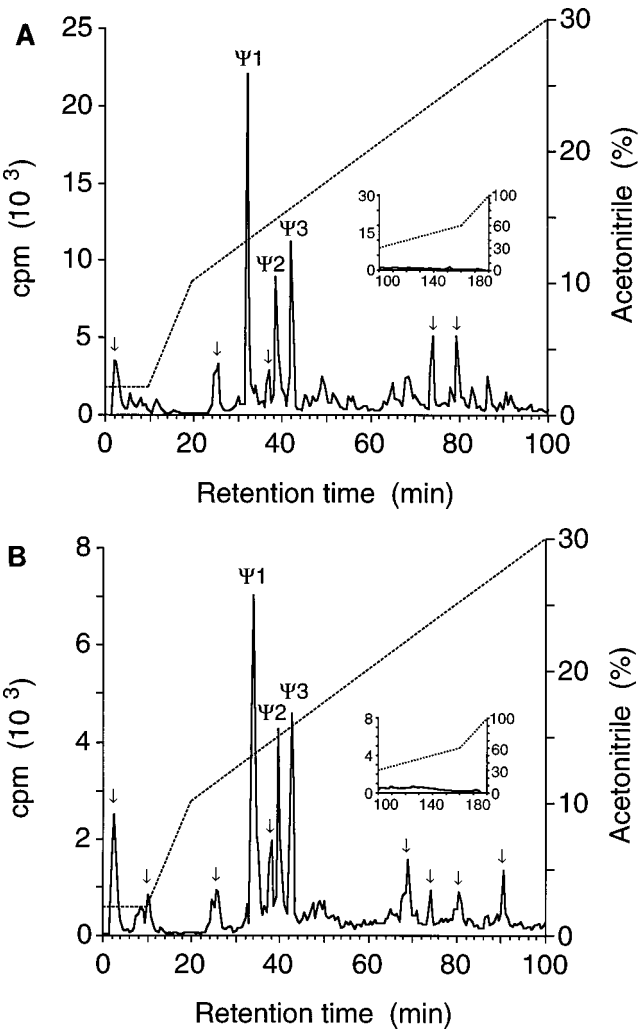

FIG. 3. HPLC phosphopeptide profiles of PKR proteins digested with chymotrypsin. (A) *trans* phosphorylation of His-PKR(K296R) by PKR(Wt). (B) Autophosphorylation of PKR(Wt). His-PKR(K296R) and PKR(Wt) were mixed at a molar ratio of 10:1, and [γ-^{32}P]ATP-mediated in vitro phosphorylation was carried out by addition of 0.25 volume of 5× reaction buffer (10 mM magnesium acetate, 0.1 mM ATP, 50 to 60 μCi of [γ-^{32}P]ATP per ml) containing 10 μg of poly(rI)-poly(rC) per ml. The final concentration of poly(rI)-poly(rC) (2.0 μg/ml) was optimized for large-scale phosphorylation reaction mixtures. After incubation at 30°C for 30 min, proteins were fractionated by SDS–10% PAGE and electroblotted onto nitrocellulose. Radioactive bands corresponding to either His-PKR(K296R) or PKR(Wt) were excised and digested in situ with chymotrypsin as described by Aebersold (1). Chymotrypsin digestion products were fractionated on a C_{18} reversed-phase HPLC column (Waters Delta Pak C_{18}) with a 2 to 100% acetonitrile gradient in 0.1% trifluoroacetic acid. Fractions were collected every 0.5 min during the first 100 min and every 2 min thereafter (inset). Fractions were directly measured (Cerenkov method) for radioactivity with a Beckman LS1801 scintillation system. The amount of ^{32}P present in the fractions was plotted against the retention time. The acetonitrile concentration gradient is shown by the broken line. The positions of the three major peaks, Ψ1, Ψ2, and Ψ3, are indicated. The minor peaks mentioned in the text are marked by arrows.

The phosphopeptide profile of purified His-PKR(K296R) that had been treated with bacteriophage λ phosphatase prior to the in vitro *trans* phosphorylation by PKR(Wt) (data not shown) was not significantly different from that of His-PKR(K296R) that had not been treated with λ phosphatase (Fig. 3A). Therefore, it appears that His-PKR(K296R) expressed with the baculovirus system was not significantly phosphorylated by an endogenous insect cell kinase on sites that were phosphorylated in vitro by PKR(Wt).

Somewhat surprisingly, the chymotryptic phosphopeptide profile of autophosphorylated PKR(Wt) also showed three major peaks (Ψ1, Ψ2, and Ψ3) with retention times of 34.0, 39.5, and 42.5 min, respectively (Fig. 3B). Most likely, these three peaks from PKR(Wt) correspond to the three major His-PKR(K296R) peaks, Ψ1, Ψ2, and Ψ3 (Fig. 3A). The relative magnitudes of the three peaks were similar in the two phosphopeptide profiles, suggesting that the extent of intermolecular *trans* autophosphorylation of His-PKR(K296R) was similar to that of PKR(Wt) autophosphorylation. Furthermore, several minor phosphopeptide peaks were also detected with PKR(Wt), as was observed with His-PKR(K296R), for example, peaks 2.5, 25.5, 38.5 (K296R; 37.0), 74.0, and 80.5 (K296R; 79.5). These results suggest that most of the sites that were *trans* autophosphorylated in His-PKR(K296R) by PKR(Wt) were similar, if not identical, to the sites of autophosphorylation in PKR(Wt).

The degree of similarity of the phosphorylation sites of PKR(Wt) and His-PKR(K296R) was also assessed by digestion with *Staphylococcus aureus* V8 protease. The V8 phosphopeptide profiles of *trans*-phosphorylated His-PKR(K296R) and autophosphorylated PKR(Wt) were similar (data not shown), further supporting the notion that the phosphorylation sites of His-PKR(K296R) and PKR(Wt) are very similar, if not identical.

Conclusions. Because His-PKR(K296R) possesses no de-

tectable intrinsic protein kinase activity (11, 24), *trans* autophosphorylation by PKR(Wt) involves only intermolecular events, whereas autophosphorylation of PKR(Wt) likely includes both intramolecular and intermolecular events. Herein we provide evidence based on phosphopeptide analyses that the major in vitro phosphorylation sites of His-PKR(K296R) and PKR(Wt) are very similar, if not identical. Therefore, it is likely that the autophosphorylation of PKR(Wt) which leads to activation can occur by a mechanism that involves, in part, intermolecular phosphorylation events. Determination of the specific amino acid residues phosphorylated in PKR is of considerable importance. However, attempts involving both direct chemical analysis and mutagenesis have been hindered by the unusually high serine-threonine content of PKR (16, 22).

The observation that excess His-PKR(K296R) inhibited both autophosphorylation of PKR(Wt) and *trans* phosphorylation of His-PKR(K296R) by activated PKR(Wt) may be relevant to the observed dominant negative behavior of the K296R mutant observed within intact cells. For example, the PKR(K296R) mutant enhances the expression of reporter protein synthesis in transfected cells (2, 9) and rescues *Saccharomyces cerevisiae* from the growth suppression mediated by PKR(Wt) (5). The facts that His-PKR(K296R) inhibited both the autophosphorylation and *trans* phosphorylation activities of PKR(Wt) and that this inhibition could be relieved by addition of higher concentrations of activator RNA suggest that one of the mechanisms contributing to the dominant negative property of PKR(K296R) appears to be its competition with endogenous PKR(Wt) for binding of activator RNAs. This conclusion is supported by the observations that unrelated RNA-binding proteins, such as the vaccinia virus E3L protein and the reovirus σ3 protein, can increase the synthesis of reporter proteins in transfected cells (9) and reverse the interferon-sensitive phenotype of the vaccinia virus E3L mutant (3).

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