Identification and Requirement of Three Ribosome Binding Domains in dsRNA-Dependent Protein Kinase (PKR)†

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Received May 27, 1998; Revised Manuscript Received July 31, 1998

ABSTRACT: The interferon-inducible, double-stranded (ds) RNA-dependent protein kinase (PKR) regulates protein synthesis initiation by phosphorylating the α-subunit of eukaryotic translation initiation factor 2 (eIF-2). The amino-terminal half of PKR contains two dsRNA binding domains, and the kinase domain resides in the carboxy-terminal half of the protein. PKR is a ribosomal-associated protein. In this report, we provide evidence that PKR contains three ribosome interaction sites, two that are localized in each of the dsRNA binding domains and one that is localized in the kinase domain. All three domains can associate with polysomes independently. The ribosome association of the dsRNA binding domains requires dsRNA binding activity. Ribosome interaction of either the individual or the combined dsRNA binding domains was disrupted by 0.1 M KCl. In contrast, the ribosome interaction of intact PKR and the isolated kinase domain was largely resistant to 0.5 M KCl. These results indicate that all three domains of PKR contribute to the high-affinity ribosomal association. After dissociation of polysomes with EDTA, both intact PKR and the isolated kinase domain were primarily associated with the 60S ribosomal subunit. Coexpression of the adenovirus VAI RNA, an RNA polymerase III gene product that binds and inactivates PKR, disrupted ribosomal association of intact PKR, but not of the isolated PKR kinase domain. The results support a model where VAI RNA induces a major conformational change in PKR to prohibit ribosome association of all interaction sites. In contrast, other inhibitors of PKR including vaccinia virus E3L and K3L gene products, and the HIV trans-activating response (TAR) element binding protein TRBP, did not disrupt ribosome association of PKR. The results suggest a novel mechanism by which viral RNAs may inactivate PKR through disrupting ribosome association.

The double-stranded (ds) RNA-dependent protein kinase (PKR)[1] is a major mediator of the cellular response to stress, such as growth factor depletion, heat shock, and virus infection. Upon stress induction, PKR becomes activated through autophosphorylation and dimerization. Activated PKR phosphorylates the α-subunit of the eukaryotic translation initiation factor 2 (eIF-2α) on serine residue 51, a modification that eventually inhibits protein synthesis initiation (*1*−*3*). PKR activation and subsequent eIF-2α phosphorylation is the primary mechanism that prevents viral replication as part of the interferon anti-viral response (*4*). Recently, it has become evident that PKR may also play a critical role in regulation of cell growth (*5*−*7*), dsRNA-dependent transcriptional regulation (*8*−*11*), regulation of differentiation (*12, 13*), induction of apoptosis (*14*−*16*), and suppression of cell transformation (*6, 7*).

The catalytic kinase domain of PKR is contained within the C-terminal half of the protein and includes a lysine at residue 296 that is required for ATP hydrolysis (Figure 1A). The N-terminus of PKR contains two dsRNA binding domains, each which is comprised of a dsRNA binding motif rich in basic amino acids that is conserved among many dsRNA binding proteins (*17*−*20*). Between the two dsRNA binding domains and the catalytic kinase domain is a third region that is rich in basic amino acids (residues 243−272) and is required for catalytic activity of the kinase (*21, 22*).

PKR is synthesized in a latent form that requires activation by dsRNA. Numerous studies support that dsRNA binding to PKR induces dimerization with subsequent trans-autophosphorylation and activation of the eIF-2α kinase activity (*23*−*29*). The activation curve for dsRNA is bimodal where low concentrations of dsRNA activate and high concentrations of dsRNA actually inhibit PKR activation. The structural requirements for the intermolecular interaction between dsRNA and PKR have been extensively studied. Short RNA duplexes of 16 bp are capable of binding PKR; however, the binding efficiency and activation increase with length up to 85 bp (*30*−*34*). Each of the dsRNA binding domains binds dsRNA independently, although the first domain contributes more to the stability of the RNA−protein complex. Mutation of the conserved lysine at residue 64 to glutamic acid (K64E) within the first dsRNA binding motif

† Portions of this work were supported by NIH Grant 1RO1 AI42394 (R.J.K.).

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[1] Abbreviations: PKR, double-stranded RNA-dependent protein kinase; PKRΔD1, PKR without dsRNA binding domain 1 aa 98−551; KD, PKR kinase domain aa 228−551; KDΔD3, PKR kinase domain aa 264−551; BD, PKR dsRNA binding domains aa 1−243; D1, PKR dsRNA binding domain 1 aa 1−123; D2, PKR dsRNA binding domain 2 aa 98−243; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; RBS, ribosome binding solution; PBS, phosphate-buffered saline.

S0006-2960(98)01472-X CCC: $15.00
Published on Web 09/09/1998

significantly reduced dsRNA binding (*20, 29, 34*).

PKR is ubiquitously expressed in mammalian cells, and immunofluorescence analysis demonstrated that PKR is associated with the surface of the rough endoplasmic reticulum as well as nucleolus (*35*−*37*). This is consistent with the observation that PKR is a ribosome-bound protein that can be washed away in high-salt buffers (*24, 38, 39*). Recent studies on the expression of human PKR in yeast suggest that the dsRNA binding domain of PKR mediates association with the 40S ribosomal subunit (*40*).

Various inhibitors of PKR-mediated eIF-2α phosphorylation exist within interferon-resistant viral genomes including the small RNA polymerase III transcripts encoded by adenovirus (VA RNAs I and II) and Epstein Barr virus (EBER RNAs I and II) (*41*−*43*) as well as protein products such as E3L and K3L that are encoded by vaccinia virus (*44, 45*). Whereas E3L is a double-stranded RNA binding protein that competes with PKR for binding to dsRNA, K3L bears amino acid sequence homology to the phosphorylation site on the eIF-2α subunit and competitively inhibits the interaction of PKR with the eIF-2α subunit (*46*). To date, several cellular gene products have also been characterized that inhibit PKR activity: (1) a cellular 58 kDa protein is activated upon influenza virus infection (*21, 47*); (2) a protein factor is synthesized in poliovirus-infected cells (*48*); (3) the cellular HIV TAR RNA binding protein (TRBP) binds RNAs containing specific secondary structure(s) and inhibits PKR activation (*49*); (4) a 100 kDa PKR inhibitor is associated with Ha-*ras*-mediated transformation of NIH3T3 cells (*50*); (5) La autoimmune antigen both sequesters and unwinds dsRNA (*51*); (6) a 15 kDa protein (dRF) is expressed in 3T3 F442A upon growth arrest with subsequent induction of adipocyte differentiation (*52*); and (7) a 67 kDa glycoprotein (p67) that copurifies with eIF-2 protects the eIF-2 α-subunit from inhibitory phosphorylation by eIF-2 kinases (*53*). However, to date, there is no evidence that any of the PKR antagonists affect PKR activity by altering its association with polysomes.

To study the relationship between dsRNA binding and the ribosome association of PKR, we generated a set of PKR deletion mutants. Analysis of the proteins expressed in COS-1 monkey cells identified the domains of PKR that are necessary and sufficient for ribosomal association. The results show that both dsRNA binding motifs can independently associate with ribosomes and the dsRNA binding activity is required for this association. The isolated kinase domain can also independently associate with ribosomes, and the basic region in the middle of PKR is not required for this association. The interaction was mediated primarily by the 60S ribosomal subunit. Therefore, both dsRNA binding motifs as well as the kinase catalytic domain contribute to high-affinity ribosome interaction. To further characterize the requirement for ribosomal association and activity of PKR, the effect of coexpression of known PKR inhibitors on ribosome association was studied. Of the inhibitors tested, only adenovirus VAI RNA interfered with the ribosome association of PKR. The results suggests a novel mechanism by which viral RNA inhibitors may regulate PKR activity.

EXPERIMENTAL PROCEDURES

Expression Vectors. The expression plasmid pETFVA$^-$ used in this study was previously described (*46*). It contains a transcription unit utilizing the adenovirus major late promoter and simian virus 40 (SV40) enhancer element. In addition, the vector contains the SV40 origin for replication in COS-1 cells. The pETFVA$^-$ expression vectors encoding K296P PKR (K296P), the K64E/K296P double mutant PKR (K64E/K296P), the PKR dsRNA binding domain (BD, amino acid residues 1−243), the K64E mutant BD (K64E-BD), the third basic region PKR deletion contained within the K296P mutant PKR (K296P/KDΔD3, residues 264−551), and the K296P mutant PKR isolated kinase domain (K296P/KD, amino acid residues 228−551) were described previously (*28, 29*). Where indicated, the same expression constructs were used that contained a bacteriophage T7-epitope tag in the carboxy terminus (*28*). The dsRNA binding domain 1 deletion mutant of K296P (K296PΔD1) was made by PCR using the method previously described (*29*). The sequences of the mutants were determined by the dideoxy nucleotide sequencing method (*54*). The adenovirus VAI RNA containing vector pVA-SVOD and control vector pSVOD were previously described (*55*).

DNA Transfection and Analysis of Cell Extracts and S100 Fractions. COS-1 monkey kidney cells were transfected by the DEAE-dextran procedure (*56*). For each 100 mm plate, 4 μg of plasmid DNA was used in a volume of 4 mL of transfection medium. For cotransfection, equal amounts of both plasmid DNAs were used (4 μg each). After 48 h, cells were labeled with Expre [^{35}S] [^{35}S] protein labeling mixture (100 μCi/mL; 1000 Ci/mmol; New England Nuclear Corp., Boston, MA) for 20 min in methionine- and cysteine-free minimal essential medium (GIBCO BRL, Gaithersburg, MD) followed by 1 h chase in Dulbecco's modified Eagle's medium (Life Technologies, Gaithersburg, MD) with 10% fetal calf serum, 1 mM methionine, and 1 mM cysteine. Cell extracts were prepared by harvesting the cells in 500 μL of ribosome binding solution (RBS: 10 mM KCl, 1.5 mM $MgCl_2$, and 10 mM Tris-HCl, pH 7.5) containing 0.5% NP-40. The nuclei and debris were removed by centrifugation at 5000 rpm at 4 °C in a microcentrifuge apparatus.

Cell extracts were fractionated by ultracentrifugation at 100000*g* at 4 °C in a Beckman ultracentrifuge using a swinging-bucket rotor (SW60, Beckman, Arlington Heights, IL). The supernatants were collected, and the pellets were rinsed twice with phosphate-buffered saline (PBS). The pellets were then sequentially washed with 500 μL of RBS containing 100 mM KCl (0.1 M salt wash) and 500 μL of RBS containing 500 mM KCl (0.5 M salt wash). The final pellets from the 0.5 M salt wash were resuspended in RBS. The salt washes of the S100 pellet were dialyzed against RBS in a microdialyzer (Pierce, Rockford, IL). Proportionate volumes of each sample were then analyzed by SDS−PAGE under reducing conditions (*57*). Gels were fixed in 30% methanol−10% acetic acid, prepared for fluorography by treatment with En3Hance (New England Nuclear Corp., Boston, MA), and dried. Dried gels were autoradiographed with BIOMAX MR film (Eastman Kodak, Rochester, NY). The levels of the polysome-associated proteins were quantitated using NIH-Image (Version 1.57, NIH, Bethesda, MD).

Preparation and Analysis of Polysomes. COS-1 cells were transfected and labeled with Expre [^{35}S] [^{35}S] protein labeling mixture as described above. Unless otherwise noted, the cells were harvested in 50 mM Tris-HCl, pH 7.4, 25 mM KCl, 5 mM $MgCl_2$, 1% Triton-X100, 1% sodium deoxy-

Table 1: Distribution of PKR Fragments in S100 Pellets without or with Coexpression of VAI RNA

	fragments							
	K296P	K64E/K296P	KD	BD	K64E/BD	D1	K64E/D1	D2
% in polysome (without VA)	78 ± 3	69 ± 4	82	67 ± 1	39	41 ± 6	0	28 ± 6
% in polysome (with VA)	39	54		63		35		25

cholate, and 10 μg/mL cycloheximide. The lysates were centrifuged at 10000*g* for 10 min, and the supernatants were loaded onto linear sucrose gradients made in the same buffer containing either 5 mM $MgCl_2$ or 10 mM EDTA. The samples were centrifuged at 150000*g* for 2−4 h and collected into fractions, and the absorbency at 260 nm was measured. Where indicated, aliquots (20 μL) were taken directly for SDS−PAGE or for immunoprecipitation of PKR using anti-bacteriophage T7-epitope antibody or anti-PKR polyclonal antibody (kindly provided by B. R. G. Williams) as described (*28*). Where indicated, proteins were precipitated by addition of trichloroacetic acid (TCA) to 15% and stored at −20 °C overnight. Proteins were pelleted by centrifugation at 10000*g* for 15 min followed by two washes with cold acetone. The samples were dried, resuspended in SDS−PAGE loading buffer, and analyzed by SDS−PAGE under reducing conditions as described above.

Western immunoblot analyses were performed by the enhanced chemiluminescence (ECL) detection method (Amersham Life Sciences Corp.). Proteins from sucrose gradient fractions were subjected to SDS−PAGE and transferred to nitrocellulose membranes. The membranes were blocked in Tris-HCl-buffered saline−Tween 20 (TBST) containing 5% skim milk for 1 h. The membranes were then incubated in the presence of primary polyclonal antibody against human PKR (kindly provided by Bryan R. G. Williams) or antibody against the bacteriophage T7-epitope (Novagen Corp.) for 1 h followed by three washes with TBST. The membranes were then incubated in 5% skim milk containing appropriate secondary antibody for 45 min followed by three washes with TBST. Finally the nitrocellulose membranes were developed in the presence of developer supplied by Amersham Life Science Corp.

RESULTS

PKR Contains Three Ribosome Interaction Domains. To study the interaction of PKR with polysomes, we used a series of point mutants and deletion mutants of PKR (Figure 1A). The point mutants studied were K64E in the first dsRNA binding motif that is defective in dsRNA binding activity (*20*, *29*, *34*) and K296P within the kinase domain that is catalytically inactive (*7*). The K64E mutation was studied to evaluate the requirement for dsRNA binding in polysome association. The K296P mutation was introduced to destroy PKR activity to enable the synthesis of the PKR variants. The PKR fragments studied were the dsRNA binding domain D1 (residues 1−123), the dsRNA binding domain D2 (residues 98−243), the intact dsRNA binding domain BD (residues 1−243), the intact K296P mutant kinase domain K296P-KD (residues 228−551), PKR deleted of the first dsRNA binding domain K296PΔD1(residues 98−551), and the inactive catalytic domain that is deleted of the third basic region K296P-KDΔD3 (residues 264−551).

Expression vectors encoding each PKR molecule were transiently transfected into COS-1 cells, and the cells were pulse-labeled with [^{35}S]methionine/cysteine and chased for 1 h in medium containing excess unlabeled methioinine and cysteine to ensure completion of radiolabeled nascent polypeptide chains. Total cell extracts were prepared for analysis of protein expression by SDS−PAGE and autoradiography. The polysomal association of the expressed proteins was measured by analysis of the proteins cosedimenting with polysomes. Although the expression level of the different PKR mutants varied to some degree, it was possible to detect the synthesis of each PKR protein in the total cell extract as polypeptides migrating with the expected molecular weight (Figure 1B). Extracts were fractionated by centrifugation at 100000*g*, and the S100 supernatant and the S100 pellet samples were analyzed by SDS−PAGE. Comparison of S100 supernatant with the S100 pellet demonstrated that the amount of each of the expressed PKR proteins, except for K64E-D1, was greatly enriched in the pellet fraction that contained polysomes (Figure 1C,D). The absence of K64E-D1 in the S100 pellet demonstrated specificity in detection of the different PKR mutants and fragments in the S100 pellet. Either dsRNA binding domain alone (D1 or D2) was independently able to interact with polysomes (Figure 1D, lanes 8, 10). Deletion of either D1 (K296PΔD1), the dsRNA binding domain (K296P-KD), or the dsRNA binding domain with D3 (K296P-KDΔD3) did not destroy the ability for PKR to associate with polysomes, although these molecules were expressed at a lower level than the intact PKR (Figure 1D, lanes 3−5). The amount of protein detected in the S100 pellet was quantitated by comparison to the amount of protein in the S100 supernatant (Table 1). The proportion of precipitated D2 (30%) was significantly reduced compared to BD (69%) and D1 (47%) (Figure 1C and Figure 1D, lane 10 vs lanes 6 and 8). In addition, mutation of the dsRNA binding activity by mutation of K64E had the most dramatic effect in reducing the amount of protein in the polysomal precipitate for the D1 fragment (not detectable), an intermediate effect for the BD fragment (33%), and a lesser effect for the intact PKR molecule (compare K296P with the K64E/K296P double mutant). We interpret these results as suggesting that each of the two motifs of the dsRNA binding domain, as well as the kinase domain of PKR, can associate with polysomes independently. In addition, ribosome association of the dsRNA binding motif D1 requires dsRNA binding activity, and the requirement for dsRNA binding is less significant within the intact PKR molecule.

All Three Subdomains of PKR Contribute to High-Affinity Ribosomal Association. To characterize the ribosome affinity for each of the domains of PKR, the S100 pellets were washed with increasing concentrations of salt (Figure 2A−E). For BD, D1, and D2, most of the PKR that coprecipitated with polysomes was released in the 0.1 M salt wash (Figure 2C, lanes 3−5). However, intact K296P as well as a significant amount of the isolated kinase domain K296P-KDΔD3 remained in the S100 pellet fraction after the 0.5

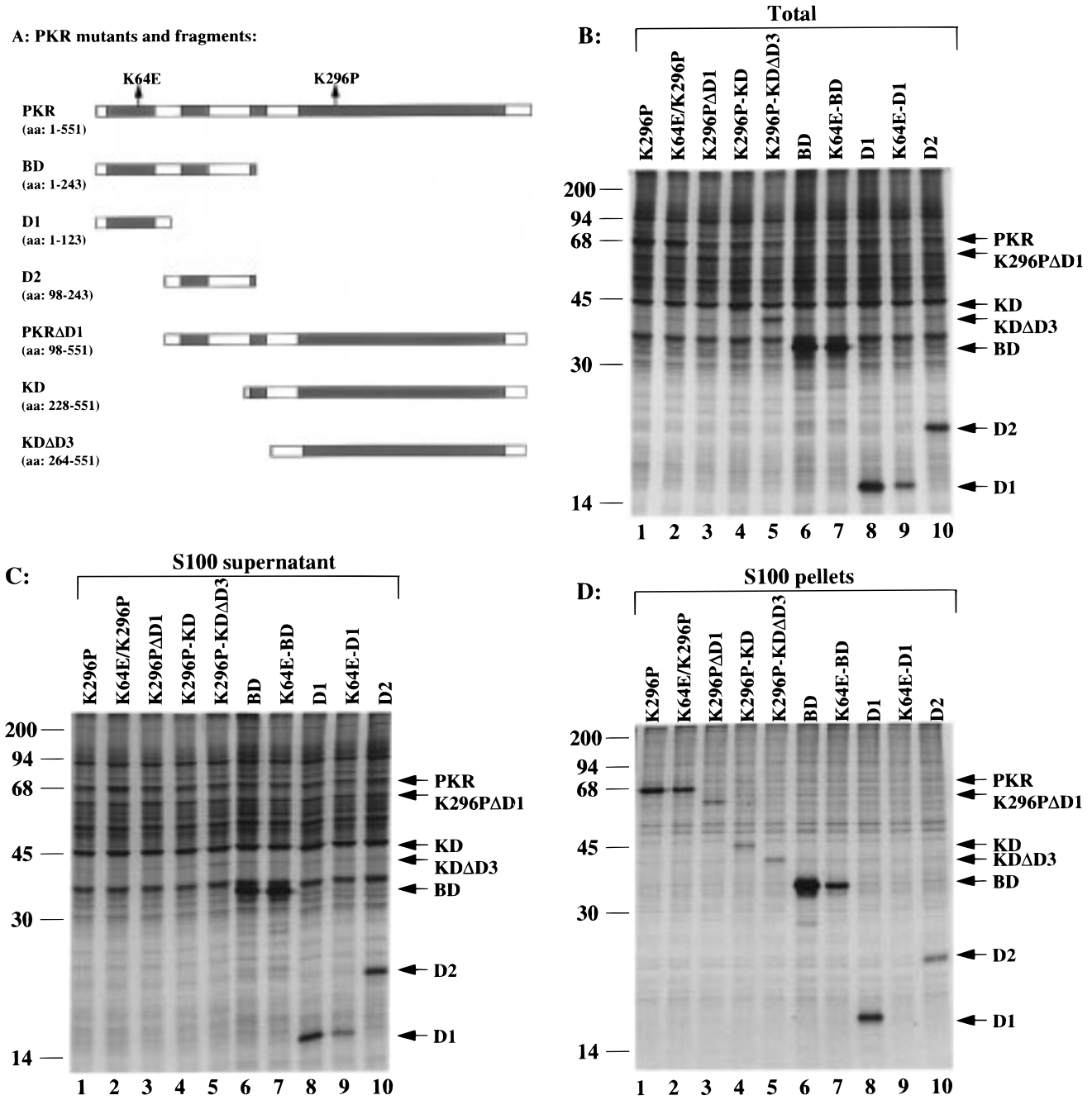

FIGURE 1: PKR contains three polysome association sites. The PKR mutants and fragments used in this study are depicted (A). The shaded areas represent structural motifs presented in the order D1, D2, D3, and KD. COS-1 cells were transfected with PKR mutants (as indicated) contained in a mammalian expression vector pETFVA$^-$ and analyzed as described under Experimental Procedures. Panel B: Analysis of total cell extracts. The migration positions of the PKR mutants are indicated. Panel C: Analysis of the supernatants of S100. Panel D: Analysis of the S100 precipitates.

M salt wash (Figure 2E, lane 1). These results suggest that the isolated kinase domain displays the higher affinity for polysomes than do either of the dsRNA binding domains. The results suggest that all three polysomal association sites on PKR are required for the highest affinity interaction of PKR with polysomes.

K296P-PKR and K296P-KD Associate with Polyribosomes. For convenience, the above experiments measured the ability of different PKR molecules to associate with polysomes by measuring their coprecipitation with polysomes. To demonstrate that PKR and the isolated kinase domain KD were actually associated with polyribosomes, the cell lysates were centrifuged through a sucrose gradient in the presence of either 5 mM $MgCl_2$ or 10 mM EDTA. Analysis of fractions by SDS−PAGE and autoradiography demonstrated that in the presence of $MgCl_2$, K296P-PKR cosedimented with polyribosomes at the bottom of the gradient in fractions 2−5 and with monosomes in fractions 6−8 (Figure 3A). In the presence of EDTA, polysomes were disrupted into 60S and 40S ribosomal subunits. Analysis of PKR in these fractions demonstrated enrichment of PKR in the 60S ribosomal subunit fractions (*9*−*10*). The presence of PKR in these fractions was confirmed by Western blot analysis (Figure 3C).

A similar analysis of polysomes was performed with COS-1 cells that were transfected with bacteriophage T7-

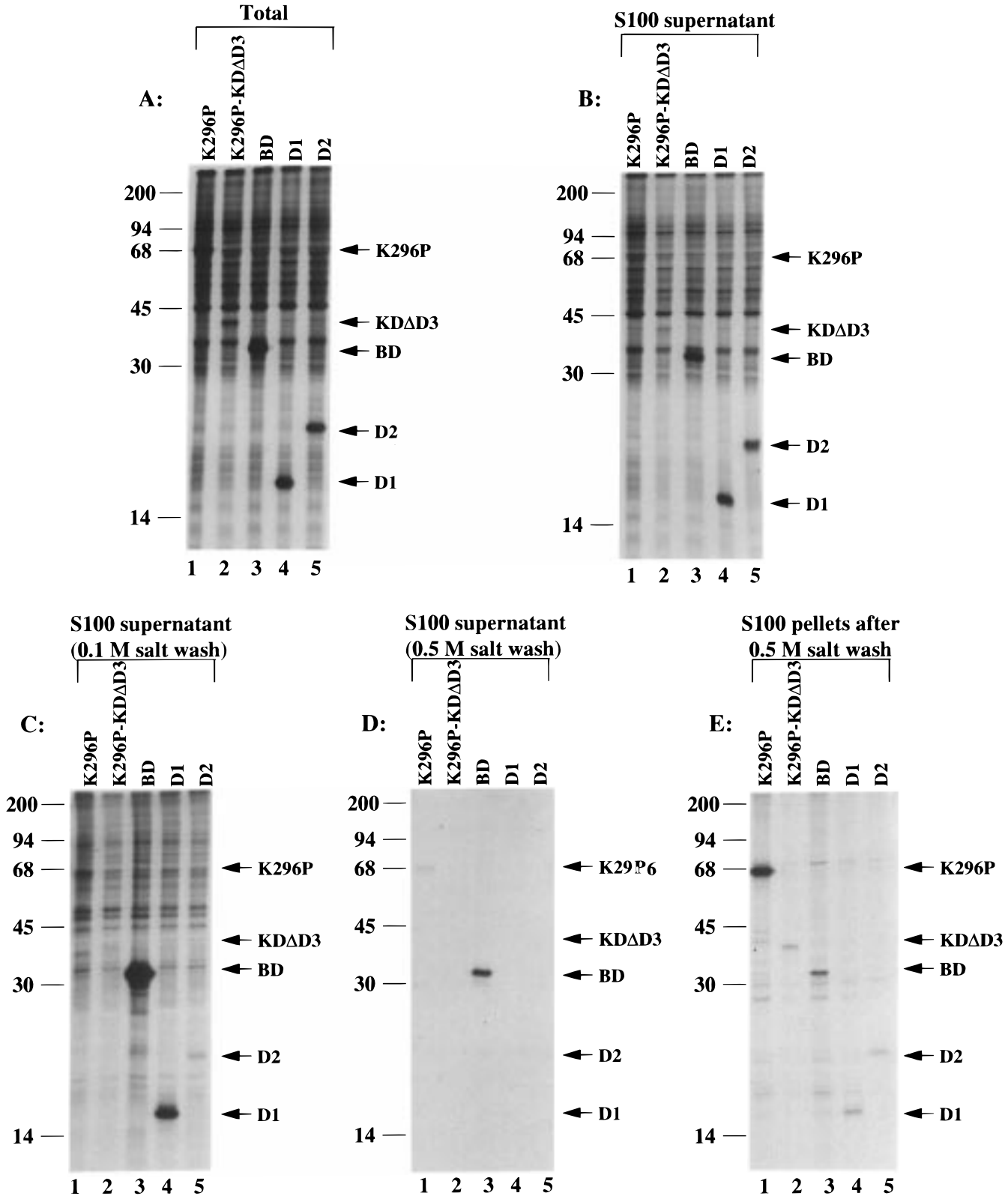

FIGURE 2: All three domains of PKR contribute to the ribosome interaction. COS-1 cells were transfected with PKR mutants and/or fragments (as indicated) and labeled with [^{35}S]methionine/cysteine as described under Experimental Procedures. The cells were lysed with 0.5 mL of RBS containing 0.5% NP-40. S100 was then prepared from the cell extract. The pellets of S100 were resuspended in 0.5 mL of RBS containing 0.1 M KCl (0.1 M salt wash). S100 was prepared again, and the pellets were resuspended in 0.5 mL of RBS containing 0.5 M KCl (0.5 M salt wash). The resuspended cell extracts were centrifuged at 100000*g* again, and the pellets were resuspended in RBS. Panel A: Total cell extracts of the transfected COS-1 cells. Panel B: Supernatants of S100. Panel C: The 0.1 M salt wash. Panel D: The 0.5 M salt wash. Panel E: Resuspended pellets of the 0.5 M salt wash.

epitope-tagged mutant K296P-KD and then labeled with [^{35}S]-methionine and cysteine. Analysis of fractions by SDS−PAGE and autoradiography demonstrated that in the presence of $MgCl_2$, K296P-KD was associated with polyribosomes in fractions 3−5, although to a lesser extent than intact K296P-PKR, and monosomes in fractions 6−8. Upon

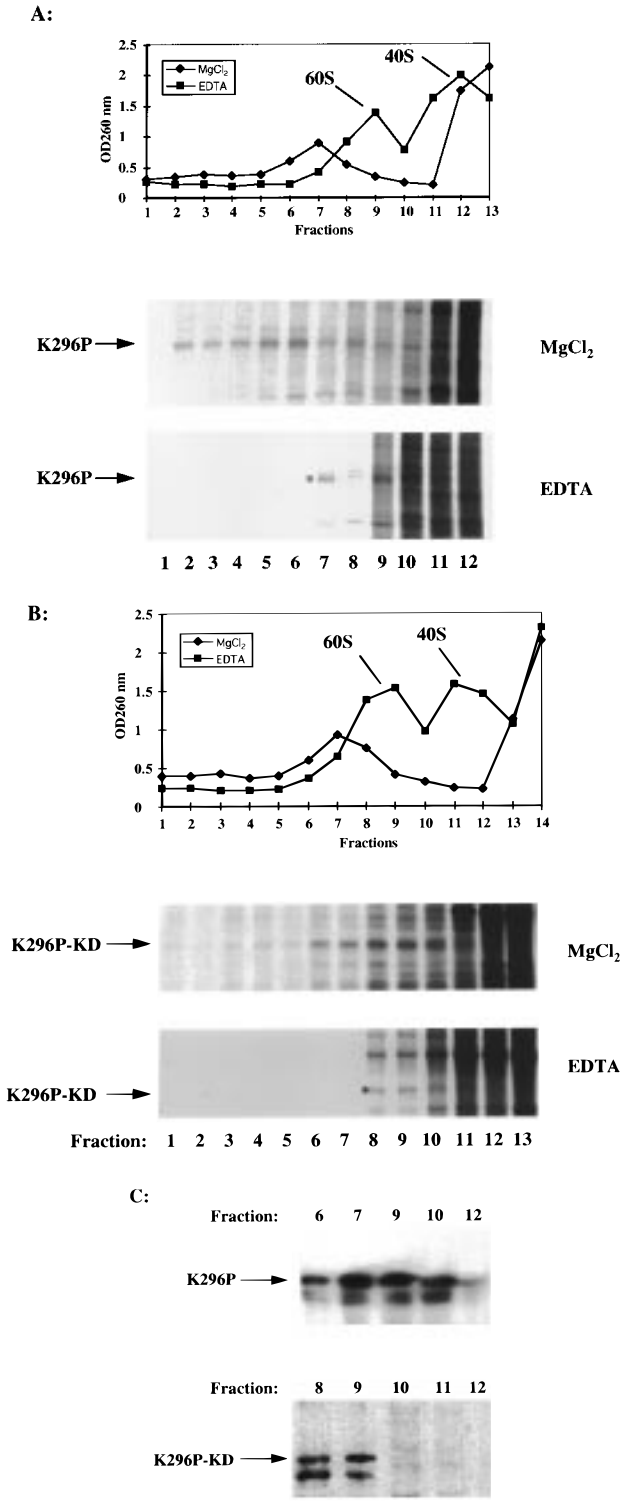

FIGURE 3: K296P-PKR and K296P-KD are associated with the 60S ribosomal subunit. COS-1 cells transfected with K296P-PKR (panel A) or K296P-KD (panel B) were labeled and prepared for analysis of polyribosomes by centrifugation on a 7−50% (w/w) sucrose gradient with either $MgCl_2$ or EDTA as described under Experimental Procedures. Fractions were collected from the bottom of the tube, and the absorbency (OD_{260}) was measured. The sucrose gradient sedimentation profile is shown on the top of panels A and B. Aliquots of fractions were subjected to SDS−PAGE and autoradiography. The asterisk indicates migration of K296P-PKR and K296P-KD. Aliquots of fractions from the gradients in the presence of EDTA were analyzed by Western blot analysis using either anti-PKR antibody (for K296P-PKR) or anti-T7 antibody (for K296P-KD).

EDTA disruption of the polyribosomes and monosomes, the K296P-KD was associated primarily with the 60S ribosomal subunit (fractions 9−10) and not the 40S ribosomal subunit (fractions 11−12). The interaction of K296P-KD with the 60S ribosomal subunit was also confirmed by Western blot analysis using anti-T7-epitope antibody where K296P-KD was detected in fractions 8 and 9, but not in fractions 10−12. These results demonstrate that both intact PKR and the isolated kinase domain preferentially associate with the 60S ribosomal subunit.

Adenovirus VAI RNA Disrupts PKR Interaction with Ribosomes. To study the relationship between PKR interaction with ribosomes and PKR activity, we asked whether any known inhibitors of PKR activity can dissociate PKR from the ribosomes. Analysis of cells that were cotransfected with the K296P-PKR expression vector and a vector encoding adenovirus VAI RNA demonstrated that the majority of PKR was not associated with the polysomes. There was a significant increase in the amount of K296P-PKR detected in the S100 supernatant (Figure 4A, lane 6, vs Figure 3B, lane 6). These are the identical conditions under which we previously demonstrated that expression of VAI RNA can stimulate translation and inhibit eIF-2α phosphorylation (*29*). In contrast, cotransfection of adenovirus VAI RNA did not increase the amount of isolated dsRNA binding domain BD, D1, or D2 (Figure 4A, lanes 8−10, vs Figure 3B, lanes 8−10) or the isolated kinase domain (Figure 4A, lane 7, vs Figure 3B, lane 7) detected in the S100 supernatant. Cotransfection with either of the PKR inhibitors vaccinia virus E3L, vaccinia virus K3L, or cellular TRBP, or the dsRNA binding domain BD, or the PKR mutant substrate eIF-2α Ser51Ala did not interfere with the polysome association of intact K296P or any of the isolated dsRNA binding domains (data not shown), also under conditions known to stimulate translation and inhibit eIF-2α phosphorylation (*46*, *49*).

To further characterize the effect of adenovirus VAI RNA expression on PKR localization, sucrose gradient centrifugation was performed to quantitate PKR polysome association. COS-1 cells were transfected with K296P-PKR in the presence of either vector alone or vector expressing VAI RNA. Cell lysates were prepared, and polysomes were fractionated on a sucrose gradient. Fractions were taken for analysis of total radiolabeled cell protein and for immunoprecipitation of K296P-PKR with anti-T7 antibody that recognizes an epitope tagged to the carboxy terminus of the PKR expression construct. Analysis of the total cell extract detected K296P-PKR migrating at 69 kDa, that was not present in cells that did not receive the K296P-PKR expression vector (Figure 5, panels A−C). Analysis by immunoprecipitation demonstrated that the presence of VAI RNA displaced PKR association with the polysomes so that the majority of K296P-PKR migrated at the top of the sucrose gradient. Quantitation of these results demonstrated that in the presence of VAI RNA, K296P-PKR was reduced 3.6-fold in fractions 1−4 and was increased 3.2-fold in fractions 6−8 (Table 2). These results demonstrate a significant shift in the polysomal association of PKR by VAI RNA.

To evaluate the dsRNA binding requirements in PKR for VAI RNA-induced displacement from the polysomes, we studied the effect of VAI RNA on the ribosome interaction

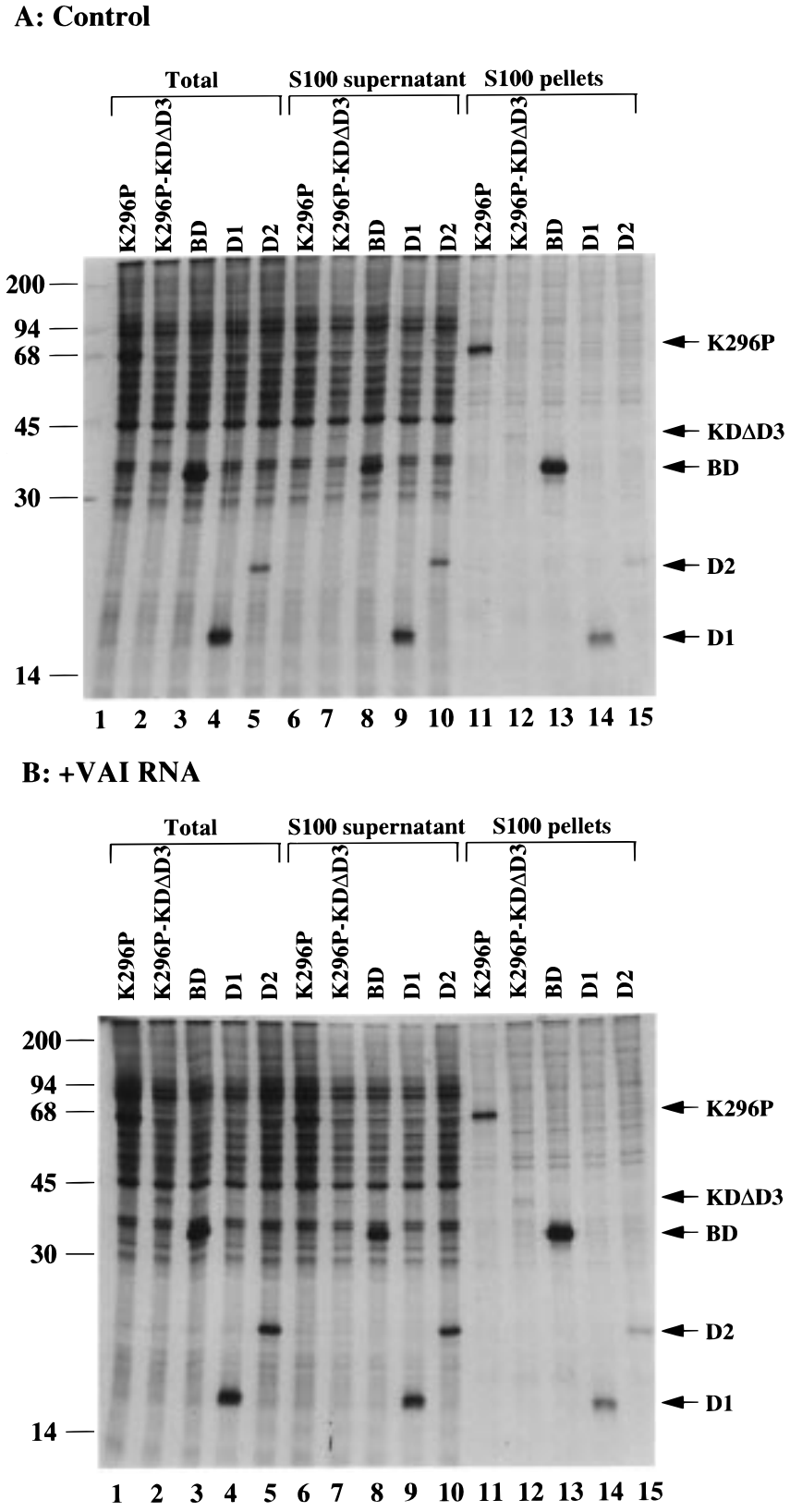

FIGURE 4: VAI RNA interferes with ribosomal association of PKR. COS-1 cells were cotransfected with the PKR expression vector indicated in the presence of an adenovirus VAI RNA containing vector pVA-SVOD (panel B) or the control vector pSVOD (panel A). Cell extracts were prepared from metabolically labeled cells and fractionated by centrifugation. The total cell extracts (lane 1−5), the supernatants of S100 (lane 6−10), and the S100 pellets (lane 11−15) were analyzed by SDS−PAGE.

of PKR deleted of the dsRNA binding domain and of PKR containing the K64E mutation that destroys dsRNA binding. Quantitation of the amount of PKR associated with the S100 pellet compared to the amount in the S100 supernatant demonstrated that the mutation of K64E within the K296P mutant intact PKR only partially reduced the amount of PKR in the S100 pellet (Figure 6A; compare lanes 3 and 4, 9 and 10, and 15 and 16). Quantitation of these results demonstated

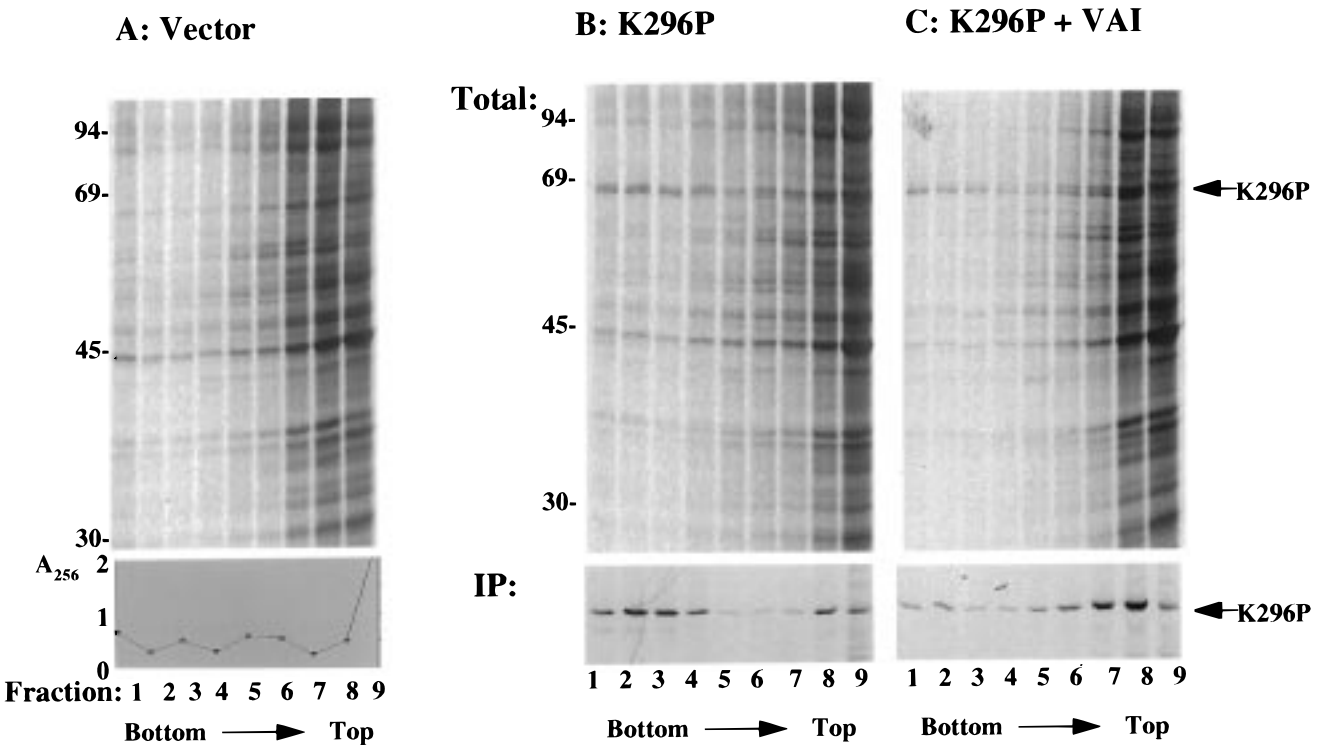

FIGURE 5: VAI RNA displaces K296P-PKR from polysomes. COS-1 cells were transfected with pETFVA$^-$ (panel A), pETFVA$^-$-K296P (panel B), and pETFVA$^-$-K296P + pVA-SVOD (panel C). At 48 h post-transfection, the cells were labeled with [^{35}S]methionine/cysteine and lysed with 0.5 mL of RBS with 0.5% NP-40. The cell extracts were fractionated by centrifugation on a 10−40% linear sucrose gradient. Equal aliquots of fractions were analyzed by SDS−PAGE and autoradiography. The optical densities of each fraction were measured at 260 nm (bottom of panel A). The expressed K296P-PKR in each fraction (panels B and C, bottom panels) was immunoprecipitated using anti-T7-epitope antibody, and the levels of K296P-PKR were quantitated using NIH-Image (Table 1).

Table 2: Distribution of K296P in the Sucrose Gradient

	fragments								
	1	2	3	4	5	6	7	8	9
intensity (units) (K296P):	1	1.20	0.27	0.30	0.34	0.84	1.66	2.20	1.35
intensity (units) (K296P+VAI):	1	3.46	2.40	0.79	0.62	0.29	0.24	0.62	0.54

a 50% reduction in the amount of K296P-PKR in the polysomes in the presence of VAI RNA (from 78% to 39%) compared to a 20% reduction in the amount of K64E/K296P-PKR (from 69% to 54%) (Table 1). In addition, VAI RNA apparently had little effect on the amount of dsRNA binding domain-deleted PKR KD associated with the S100 pellet, although this analysis could not significantly detect K296P-KD associated in the S100 supernatant (Figure 6A, lanes 11, 12, 17, 18). To quantitate the amount of K296P-KD associated with the S100 pellet, PKR was immunoprecipitated from the supernatant and pellet fractions. The results demonstrated that the majority of K296P-KD was detected in the S100 pellet fraction (Figure 6B). These results support the conclusion that displacement of PKR from the polysomes by VAI RNA required the dsRNA binding activity of PKR.

DISCUSSION

The activation of PKR from a latent state is initiated by binding to dsRNA. Upon binding to dsRNA, PKR undergoes dimerization and autophosphorylation. The requirements for dsRNA binding to PKR have been intensively studied through mutagenesis of the PKR dsRNA binding domain as well as dsRNA molecules, such as adenovirus VAI RNA (*30*−*32*). Once activated, PKR phosphorylates the α-subunit of eIF-2 which forms a 43S initiation complex with GTP, tRNA, mRNA, and the 40S ribosomal subunit. PKR is a polysomal associated protein (*38*, *39*). However, little is known about the relationship between its polysomal association, dsRNA binding, and activation. In this study, an in vivo COS-1 cell transfection system was used to study the polysomal association of PKR. Initially polysomal association was monitored by measuring the ability of different PKR molecules to coprecipitate with polysomes. Polysomal association was confirmed for K296P mutant PKR and for the intact kinase domain KD of PKR by cosedimentation with polysomes on sucrose gradients. We studied the requirements for polysome association by studying individually expressed domains of PKR as well as the effect of mutation of K64E, within the first dsRNA binding motif that is required for high-affinity dsRNA interaction (*20*, *29*, *34*). Our results show that the majority (approximately 80%) of overexpressed catalytically inactive K296P mutant PKR was associated with polysomes. This interaction was resistant to a 0.5 M salt wash, consistent with the requirement for 0.8 M KCl to release PKR from ribosomes (*58*). This demonstrates that PKR does not require the kinase activity for the high-affinity polysome association. The two isolated dsRNA binding domains of PKR, D1 and D2, either separately or together in BD, as well as the isolated Ser/Thr kinase domain alone were able to independently associate with polysomes. The binding of the isolated dsRNA binding domain D1 to polysomes required dsRNA binding activity since K64E-D1 did not detectably bind polysomes. In addition, the affinity of polysomal association was signifi-

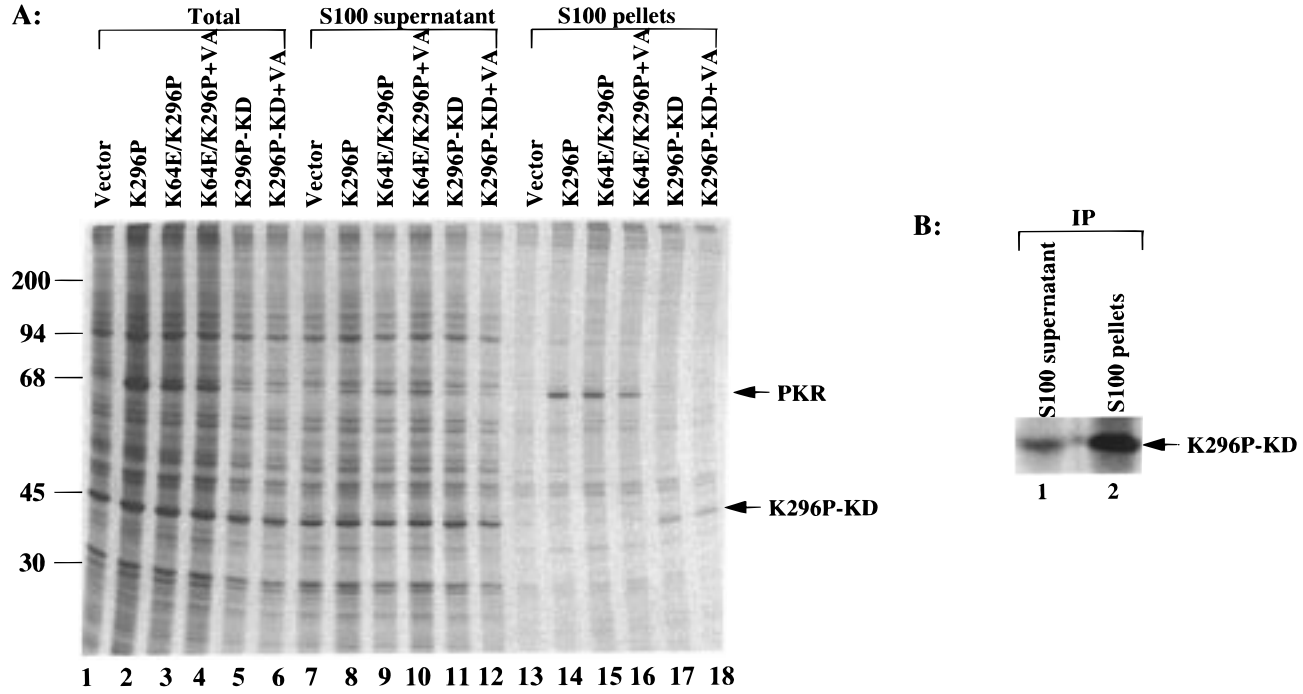

FIGURE 6: dsRNA binding is required for VAI RNA-mediated displacement of PKR from polysomes. K64E/K296P double mutant PKR and K296P-KD expression vectors were cotransfected with either pSVOD or pVA-SVOD into COS-1 cells. The transfected cells were labeled with [^{35}S]methionine/cysteine, and cell extracts were prepared and fractionated by centrifugation as described under Experimental Procedures. Aliquots of total, supernatant, and pellet fractions were analyzed by SDS−PAGE. Panel A: Analysis of total proteins in total cell extracts, the supernatants of S100, and the pellets of S100. Panel B: Immunoprecipitation of K296P-KD from the supernatants of S100 and the pellets of S100 for quantitation.

cantly reduced for K64E-BD compared to BD, suggesting that the isolated dsRNA binding domain also requires dsRNA binding within the first dsRNA binding motif for high-affinity interaction. The detectable ribosome association of K64E-BD may be due to the dsRNA binding contributed to the dsRNA binding motif contained within D2. However, ribosome association of PKR was not solely mediated by dsRNA interactions since the isolated kinase domain KD of PKR also bound to the ribosome. In fact, the isolated KD displayed an interaction that was largely resistant to 0.5 M KCl, whereas the dsRNA binding domain interactions were largely destroyed by 0.1 M KCl. Finally, deletion of the third basic region that is important for autophosphorylation did not reduce polysome association. The most logical conclusion from these observations is that all three domains contribute to the interaction of PKR with the ribosome. We propose that the dsRNA binding domain of PKR interacts with some structured RNA that is a component of the ribosome and the kinase domain interacts with a separate site on the ribosome.

Our results confirm certain aspects and extend recent observations on the interaction of human PKR with yeast ribosomes (*40*). These studies demonstrated that the dsRNA binding domain of PKR was necessary and sufficient for interaction with the 40S ribosomal subunit. However, Zhu et al. did not directly study the interaction of the isolated kinase domain in the absence of the dsRNA binding domains, as we have presented here. Our findings differ significantly in that the isolated PKR kinase domain, deleted of the dsRNA binding domains, was able to bind ribosomes with high affinity. In addition, we have observed that both intact PKR and the isolated kinase domain interact primarily with the 60S ribosomal subunit. Our results demonstrate that high-affinity interaction, measured by resistance to 0.5 M KCl, can be mediated by interaction with the kinase domain. We expect that the difference in the results stems from differences in the interaction of PKR with yeast and mammalian ribosomes. We propose that an additional protein−protein interaction between a component of the mammalian 60S ribosome and PKR is not conserved with the yeast ribosome.

Numerous inhibitors of PKR have been reported that inhibit PKR activity through different mechanisms. To date, there is no report showing any of these inhibitors deactivates PKR by interfering with polysomal association of PKR. Polysomal association of PKR was not affected by expression of the vaccinia virus PKR inhibitors E3L or K3L, or the cellular gene product TRBP. However, expression of adenovirus VAI RNA interfered with the polysome association of PKR. As previously reported, E3L and TRBP are dsRNA binding proteins that likely inhibit PKR activity through binding and sequestering dsRNA PKR activators in the cell (*46*, *49*). The vaccinia virus product K3L likely interacts with the substrate binding site of PKR to prevent PKR interaction with the wild-type eIF-2α. In contrast, adenovirus VAI RNA binds to the dsRNA binding site within PKR and prevents its activation by dsRNA (*4*). The ability of VAI RNA to displace PKR from the ribosome required that the dsRNA binding domain be present on PKR, consistent with the interaction between VAI RNA and the dsRNA binding domain. Interestingly, VAI RNA significantly reduced the interaction of intact PKR with the ribosome but had little effect on ribosome interactions of the isolated dsRNA binding domain or the isolated kinase domain. The inability of VAI RNA to significantly displace the dsRNA binding domain deleted of the kinase domain from polysome association may be due to a higher affinity

of the isolated dsRNA binding domain for the ribosome than for VAI RNA. These results suggest that the binding of VAI RNA to PKR induces a significant conformational change in PKR to reduce its affinity for ribosome interaction. This conformational change may involve dimerization. Previous studies suggested that ribosome-associated PKR is a monomer, whereas free PKR is a dimer (*24*). In addition, recent structural information suggests that the HIV-1 trans-activating region RNA, an RNA molecule that binds PKR, can induce dimerization of PKR (*59*). It is possible that induced dimerization coincides with dissociation from the ribosome.

Expression of catalytically inactive mutants of PKR can transform NIH3T3 cells (*6*, *7*). Two mechanisms are proposed to mediate the trans-dominant inhibition of the endogenous PKR activity under conditions of expression of the mutant PKR. First, PKR was proposed to form inactive heterodimers with endogenous PKR. Alternatively, PKR was proposed to bind and sequester dsRNA activators, thereby preventing PKR activation. Our results presented here suggest that overexpression of an inactive PKR molecule may inhibit endogenous PKR by displacing the functional PKR from the ribosome. Further experiments are required to test this hypothesis.

Studies have localized PKR to the rough endoplasmic reticulum in the cytosol and to the nucleolus, the site of ribosome synthesis in the cell (*35*−*37*). It is most likely that these sites of localization reflect the ribosome binding properties of PKR. Studies on the interaction of another eIF-2α kinase, GCN2, show that its ribosome association is mediated by the carboxy-terminal 120 amino acid residues, a region that is not conserved in PKR (*60*). In addition, single amino acid substitutions that activate GCN2 function to the greatest extent in vivo are localized to this ribosome interaction region and suggest the importance of ribosome interaction in the regulation of GCN2 activity (*61*). Future studies should identify what ribosomal constituents are required for PKR interaction, and this may provide an approach to develop inhibitors of PKR that may prove useful to inhibit apoptosis in pathological situations.

ADDED IN PROOF

We have recently demonstrated that PKR interacts with the large ribosomal subunit protein L18 (*62*).

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BI981472H