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Regulation of the Protein Kinase PKR by the Vaccinia Virus Pseudosubstrate Inhibitor K3L Is Dependent on Residues Conserved between the K3L Protein and the PKR Substrate eIF2α

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The mammalian double-stranded RNA-activated protein kinase PKR is a component of the cellular antiviral defense mechanism and phosphorylates Ser-51 on the α subunit of the translation factor eIF2 to inhibit protein synthesis. To identify the molecular determinants that specify substrate recognition by PKR, we performed a mutational analysis on the vaccinia virus K3L protein, a pseudosubstrate inhibitor of PKR. High-level expression of PKR is lethal in the yeast *Saccharomyces cerevisiae* because PKR phosphorylates eIF2α and inhibits protein synthesis. We show that coexpression of vaccinia virus K3L can suppress the growth-inhibitory effects of PKR in yeast, and using this system, we identified both loss-of-function and hyperactivating mutations in K3L. Truncation of, or point mutations within, the C-terminal portion of the K3L protein, homologous to residues 79 to 83 in eIF2α, abolished PKR inhibitory activity, whereas the hyperactivating mutation, K3L-H47R, increased the homology between the K3L protein and eIF2α adjacent to the phosphorylation site at Ser-51. Biochemical and yeast two-hybrid analyses revealed that the suppressor phenotype of the K3L mutations correlated with the affinity of the K3L protein for PKR and was inversely related to the level of eIF2α phosphorylation in the cell. These results support the idea that residues conserved between the pseudosubstrate K3L protein and the authentic substrate eIF2α play an important role in substrate recognition, and they suggest that PKR utilizes sequences both near and over 30 residues from the site of phosphorylation for substrate recognition. Finally, by reconstituting part of the mammalian antiviral defense mechanism in yeast, we have established a genetically useful system to study viral regulators of PKR.

A common cellular mechanism for signal transduction and regulation of gene expression involves phosphorylation of protein substrates on specific serine, threonine, or tyrosine residues. A large number of protein kinases with significant amino acid sequence similarity have been identified (25), and X-ray diffraction studies have revealed that these enzymes have a common tertiary structure (64). This high degree of sequence and structural similarity among the protein kinases contrasts with their unique substrate specificities. The molecular determinants on both the kinase and the substrate that provide this specificity are not well understood. Comparison of amino acid sequences around the site of phosphorylation on protein or peptide substrates has enabled the identification of conserved sequence motifs for a number of protein kinases and led to the model that residues immediately flanking the phosphorylation site are important determinants for substrate specificity (39, 42). Studies identifying preferred substrates for a given kinase by using degenerate peptide libraries further support the importance of flanking sequences for substrate specificity (63). In addition, the X-ray structure of a cocrystal of the cyclic AMP-dependent protein kinase PKA with its pseudosubstrate inhibitor peptide PKI provided the first picture of this interaction (43). The PKI polypeptide, which has amino acid sequence similarity with the residues flanking an authentic PKA phosphorylation site, is recognized by the kinase through a number of electrostatic and hydrophobic interactions involving the conserved residues of the recognition motif (43). However, additional sequence elements, such as the SH2 domains on the STAT transcription factors (28, 61) and the kinase docking site on c-Jun (29, 35), which are located up to 30 to 40 residues from the site of phosphorylation, can also be required by some protein kinases for efficient substrate recognition and phosphorylation.

The eukaryotic translation initiation factor 2α (eIF-2α) family of protein kinases, PKR, HRI, and GCN2, are important regulators of protein synthesis in response to cellular stress conditions (reviewed in references 12 and 30). These kinases specifically phosphorylate the α subunit of the translation initiation factor eIF2 on Ser-51. The mammalian kinase PKR is part of the cellular antiviral defense mechanism, and PKR expression is stimulated when cells are treated with interferon. When activated in virally infected cells, PKR phosphorylates eIF2α, resulting in an inhibition of protein synthesis which is believed to block viral replication. In addition to its antiviral role, PKR is also an important negative regulator of cell growth, and overexpression of mutant forms of PKR can lead to malignant transformation of mammalian cells (44, 53). The heme-regulated eIF2α kinase found principally in erythroid cells, known as HRI, functions to coordinate globin and total protein production with the cellular heme levels. When heme levels are low, HRI is active and phosphorylates eIF2α, inhibiting all cellular protein synthesis. In the yeast *Saccharomyces cerevisiae*, the eIF2α kinase GCN2 is activated under condi-

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tions of amino acid or purine nucleotide starvation. The phosphorylation of eIF2α by GCN2 results in a modest inhibition of general translation and a stimulation of *GCN4* mRNA translation (reviewed in references 30 and 31). GCN4 is a transcriptional activator of amino acid biosynthetic genes, and increased translation of *GCN4* leads to increased levels of amino acid biosynthetic enzymes, enabling the cells to withstand the starvation conditions.

eIF2 functions in protein synthesis by forming a complex with GTP and the initiator Met-tRNA^{Met}. This ternary complex (eIF2 · GTP · Met-tRNA$_i^{Met}$) binds to the 40S ribosomal subunit to form a 43S preinitiation complex, which can then bind to an mRNA and scan to the initiating AUG codon. Formation of the 80S ribosome at the AUG start codon is accompanied by hydrolysis of the GTP from the ternary complex to GDP, and eIF2 is released in a binary complex with GDP. The guanine nucleotide exchange factor eIF2B exchanges the GDP on eIF2 for GTP, so that eIF2 can participate in additional translation initiation events (33). The phosphorylation of eIF2α on Ser-51 blocks the GTP-exchange reaction and converts eIF2 into a competitive inhibitor of eIF2B, resulting in an inhibition of protein synthesis (33).

The importance of phosphorylation of eIF2α as a regulatory mechanism is illustrated by the wide variety of strategies employed by viruses to inhibit PKR and maintain normal rates of protein synthesis in the infected cells (reviewed in references 36). While some viruses, including human immunodeficiency virus, are thought to inhibit expression of PKR, others express unique protein or RNA inhibitors that directly interact with PKR. The N-terminal regulatory domain of PKR contains two double-stranded RNA (dsRNA) binding domains, which may also function as dimerization domains (24, 58). Upon binding of dsRNA, the kinase is activated to autophosphorylate and then to phosphorylate eIF2α. Adenovirus expresses an RNA, termed VAI, that binds to the dsRNA binding domains of PKR and blocks activation of the kinase (51), and the hepatitis C virus NS5A protein inhibits PKR through a direct interaction with the PKR kinase domain (23), whereas influenza virus activates a latent cellular inhibitor of PKR, P58^{IPK} (48). Vaccinia virus expresses two proteins that interfere with PKR function. The E3L protein is a dsRNA-binding protein and is thought to sequester the dsRNA activators of the kinase generated during viral replication (9). The K3L protein, expressed early during vaccinia virus infections, is believed to be a pseudosubstrate inhibitor of PKR (2, 7). Vaccinia virus mutants lacking K3L or E3L are more sensitive to interferon (2), and expression of K3L or E3L in mammalian cells can prevent the translational inhibition caused by activation of PKR (15, 16). Consistent with the alternative modes of action proposed for E3L and K3L, the inhibition of PKR by the E3L protein can be relieved by increasing the amount of dsRNA, while the K3L protein inhibition of PKR is insensitive to dsRNA levels (15, 34). The K3L protein has been shown to bind directly to PKR in vitro (7, 14), and yeast two-hybrid interactions assays have localized the K3L protein binding site on PKR to the C-terminal half of the PKR kinase domain (13, 14, 22).

Previously, it has been shown that PKR expressed in yeast can phosphorylate eIF2α and regulate translation (10, 18). High-level expression of PKR in yeast is toxic due to the inhibition of general translation (10, 18), whereas, when expressed at low levels in yeast, PKR can substitute for GCN2 and stimulate translation of *GCN4* mRNA (18). Both responses to expression of PKR in yeast are dependent on an active form of the kinase that can phosphorylate eIF2α. Thus, the mutant PKR-K296R protein, which lacks kinase activity, fails to regulate translation in yeast, and substitution of Ser-51 in yeast eIF2α by Ala blocks phosphorylation of eIF2α by PKR and eliminates the regulation of general and *GCN4*-specific translation (18). The ability of PKR to regulate translation in yeast is dependent on the dsRNA binding domains in PKR, suggesting that yeast cells possess endogenous dsRNA activators of the kinase (1, 10, 20, 58). Romano et al. (58) have shown that the degree of translational control exerted by various PKR mutant proteins in yeast correlates well with their dsRNA binding activities. The fact that PKR can phosphorylate eIF2α and regulate translation in yeast cells, combined with our ability to express wild-type and mutant forms of PKR in the absence of any endogenous eIF2α kinases, makes yeast an excellent system with which to characterize the regulation and functional properties of PKR.

The amino acid sequence of the vaccinia virus K3L protein is 28% identical to the N-terminal one-third of eIF2α (3), and since both of these proteins interact with PKR, it is expected that at least some of these conserved residues constitute a PKR recognition motif. Recently, the solution structure of a repeated domain in the *Escherichia coli* ribosomal protein S1 was determined (5), and based on sequence similarities between the S1 domain and the eIF2α and K3L proteins, it was proposed that eIF2α and the K3L protein would fold into similar structures. The sequence of eIF2α is perfectly conserved from yeast to humans over 19 residues flanking the Ser-51 phosphorylation site, which has led to speculation that this sequence is important for kinase recognition. As expected for a pseudosubstrate inhibitor, the K3L protein lacks a phosphorylatable residue corresponding to Ser-51 in eIF2α; however, it is surprising that the sequence homology between the K3L protein and eIF2α does not include the residues flanking Ser-51. Instead, the greatest homology between the K3L protein and eIF2α involves a 12-amino-acid sequence located about 30 residues from the site of phosphorylation in eIF2α. In this report, we have established a genetic system in yeast to study regulation of human PKR by the vaccinia virus K3L protein. The analysis of truncation and point mutations in K3L has established the importance of the residues conserved between the K3L protein and eIF2α for the inhibition of PKR by the K3L protein. In addition, biochemical and yeast two-hybrid interaction assays have revealed that these conserved residues are critical for the physical interaction between the K3L protein and PKR. These results raise the intriguing possibility that PKR and the other eIF2α kinases use sequences far removed from the phosphorylation site for substrate recognition. We have also identified K3L variants that are more effective inhibitors of PKR function in yeast, and these mutant proteins were found to interact more strongly with PKR. In addition, the K3L protein was found to be an allele-specific inhibitor of the yeast eIF2 kinase GCN2. Our findings have established yeast as an excellent system to study regulation of the mammalian eIF2α kinases by viral and cellular regulators and provided new insights into mechanisms of substrate recognition by protein kinases.

MATERIALS AND METHODS

Plasmids. Plasmid pOS13, carrying the vaccinia virus (strain WR) K3L gene coding sequence on an *Nco*I-*Bam*HI fragment in the vector pTM1, was described previously (7). The introduction of the *Nco*I restriction enzyme site resulted in the substitution of Val for Leu at the second codon of K3L (7). An *Nco*I (filled in with Klenow enzyme)-*Bam*HI fragment from pOS13 (a kind gift of Rosemary Jagus and Orna Elroy-Stein) was inserted between the *Sma*I and *Bam*HI sites of the yeast expression vector pEMBLyex4 (8) to create plasmid pC140. In this plasmid, K3L is expressed from a hybrid yeast *CYC1* promoter containing the upstream activating sequence from the yeast *GAL1* gene (we will refer to this construct as *GAL-CYC1*). All mutant constructs were derived from plasmid pC140. To facilitate site-directed mutagenesis of the K3L gene, an *Xho*I-*Xba*I fragment from pC140, containing part of the *GAL-CYC1* promoter and the entire K3L coding sequence, was ligated to *Xho*I- and *Xba*I-digested pBluescript

TABLE 1. Strains used

Strain	Genotype	Reference or source
H1402	*Mat*α *ino1 ura3-52 leu2-3 leu2-112* (*HIS4-lacZ*) at *ura3-52*	26
H1414	*Mat*α *ino1 ura3-52 leu2-3 leu2-112 GCN2^c-E532K* (*HIS4-lacZ*) at *ura3-52*	68
H1515	*Mat***a** *ura3-52 leu2-3 leu2-112 trp1-Δ63*	A. M. Cigan
H1608	*Mat*α *ino1 ura3-52 leu2-3 leu2-112 GCN2^c-M719V-E1537G* (*HIS4-lacZ*) at *ura3-52*	57
H1609	*Mat*α *ino1 ura3-52 leu2-3 leu2-112 GCN2^c-R699W-D918G-E1537G* (*HIS4-lacZ*) at *ura3-52*	57
H1611	*Mat*α *ino1 ura3-52 leu2-3 leu2-112 GCN2^c-E532K-E1537G* (*HIS4-lacZ*) at *ura3-52*	57
H1613	*Mat*α *ino1 ura3-52 leu2-3 leu2-112 GCN2^c-E532K-E1522K* (*HIS4-lacZ*) at *ura3-52*	57
H1642	*Mat***a** *ura3-52 leu2-3 leu2-112 trp1-Δ63* (*GCN4-lacZ TRP1*) at *trp1-Δ63*	19
H1685	*Mat*α *ino1 ura3-52 leu2-3 leu2-112 GCN2^c-M719V-E1522K* (*HIS4-lacZ*) at *ura3-52*	57
H1692	*Mat*α *ino1 ura3-52 leu2-3 leu2-112 GCN2^c-M719V-E1537G* (*HIS4-lacZ*) at *ura3-52*	C. R. Vazquez
H1894	*Mat***a** *ura3-52 leu2-3 leu2-112 gcn2Δ trp1-Δ63*	This study
H1895	*Mat***a** *ura3-52 leu2-3 leu2-112 gcn2Δ trp1-Δ63* (*GCN4-lacZ TRP1*) at *trp1-Δ63*	This study
H2543	*Mat***a** *ura3-52 leu2-3 leu2-112 gcn2Δ trp1-Δ63* (*GAL-CYC1-PKR LEU2*)$_2$ at *leu2*	58
H2544	*Mat***a** *ura3-52 leu2-3 leu2-112 gcn2Δ trp1-Δ63* (*GAL-CYC1-PKR LEU2*)$_1$ at *leu2*	P. R. Romano
J46	*Mat***a** *ura3-52 leu2-3 leu2-112 SUI2-S51A gcn2Δ trp1-Δ63* (*GCN4-lacZ TRP1*) at *trp1-Δ63*	This study
J110	*Mat***a** *ura3-52 leu2-3 leu2-112 gcn2Δ trp1-Δ63* (*LEU2*)	This study
Y190	*Mat***a** *ade2-101 gal4-Δ gal80-Δ his3-200 leu2-3 leu2-112 trp1-Δ901 ura3-52 LYS2::GAL1-HIS3 URA3::GAL1-lacZ*	27

II SK$^+$ (Stratagene) to create plasmid pC214. In addition, a *Sac*I-*Xba*I fragment from pC140, containing the entire K3L coding sequence, was inserted between the *Sac*I and *Xba*I sites of a modified pBluescript plasmid, lacking the *Hin*dIII and *Sal*I sites in the polylinker (pC311), to create plasmid pC312. Silent mutations (mutations that do not alter the encoded K3L amino acid sequence) were introduced by using PCR to create unique restriction enzyme sites within the K3L coding sequences on plasmid pC312. Plasmid pC316 was constructed by using PCR to introduce a *Hin*dIII site at K57L58 (AAA · CTG to AAG · CTT) and a *Sal*I site at V70D71 (GTT · GAT to GTC · GAC). This same K3L allele was inserted into pEMBLyex4 to create plasmid pC319. The mutations Y76A (TAT to GCT), K74A (AAA to GCT), and D78A (GAT to GCT) were constructed by using the appropriate primers for PCR, and the PCR products were subcloned into pC316 between the *Sal*I and *Xba*I sites. These mutant K3L alleles were then transferred to pEMBLyex4, creating plasmids pC326 (Y76A), pC333 (K74A), and pC334 (D78A).

The same silent mutations present in pC316 were also introduced into a hyperactive mutant allele of K3L, which is described below. First, the hyperactive mutant allele of K3L containing the mutation H47R (CAT to CGT) and a silent mutation at N80 (AAT to AAC) was subcloned as a *Sac*I-*Xba*I fragment from the pEMBLyex4-derived plasmid pC407 to pBluescript, generating plasmid pC309. Following introduction of the silent mutations to generate *Hin*dIII and *Sal*I sites (creating plasmid pC337), the mutant K3L allele was transferred to pEMBLyex4 as described above to generate plasmid pC339. The *Sal*I-*Xba*I fragment from plasmid pC337 was replaced with the same fragment from plasmid pC322 (K3L-Y76A on pBluescript) to generate pC338, encoding K3L-H47R-Y76A on pBluescript. Finally, the *Sac*I-*Xba*I fragment from pC338 was transferred to pEMBLyex4, generating plasmid pC340.

To construct C-terminally truncated alleles of K3L, specific PCR primers were used to introduce stop codons in place of the codons for M84, R83, K82, Y81, and T73. These mutations were first introduced into the K3L allele on plasmid pC214, and then the mutant K3L alleles were transferred to pEMBLyex4 to generate plasmids pC220 [M84* (ATG to TAG)], pC381 [R83* (AGG to TAG)], pC382 [K82* (AAA to TAA)], pC221 [Y81* (TAC to TAA)], and pC222 [T73* (ACA to TAA)]. These same nonsense mutations were introduced into the K3L-H47R allele, and then the alleles were transferred to pEMBLyex4 to generate plasmids pC230 (M84*), pC383 (R83*), pC384 (K82*), pC232 (Y81*) and pC389 (T73*).

Plasmid pC362, encoding a His-tagged K3L protein, was constructed by using PCR to add codons for six histidine residues (CAC) to the 3′ end of the K3L coding sequence in plasmid pC140. PCR was used to construct similar His-tagged versions of K3L-H47R (pC373) and K3L-Y76A (pC374) in the vector pEMBLyex4. Plasmid pC50 was constructed by inserting the 2.7-kb *Bam*HI fragment containing the *SUI2-S51A* allele from plasmid p1098 (18, 19) into the unique *Bam*HI site of the *URA3* integrating vector pRS306 (62). Plasmids pGAD424 and pGBT9 were from Clontech, and plasmid pAD-PKR-K296R was obtained from Michael Gale and Michael Katze (University of Washington, Seattle) and was described previously (22). pAD-PKR-K296R is a derivative of the *LEU2* vector pGAD424 and encodes a fusion between the *GAL4* activation domain and the PKR-K296R protein expressed under the control of the *ADH1* promoter. Plasmids encoding fusions between the *GAL4* DNA binding domain and wild-type K3L (pC410), K3L-H47R (pC411), and K3L-Y76A (pC412) were constructed by using PCR. By using primers that introduce an *Eco*RI restriction site at the 5′ end and retain a *Bam*HI restriction site at the 3′ end, the coding sequences for K3L, K3L-H47R, and K3L-Y76A were amplified by PCR. Following amplification, the PCR products were digested with *Eco*RI and *Bam*HI and then ligated to the identically digested pGBT9. The construction of the pEMBLyex4 plasmids expressing PKR (p1420) and PKR-K296R (p1421), as well as the low-copy-number plasmid expressing PKR (p1419), has been described previously (18). All constructs generated by PCR were sequenced by the dideoxy-chain termination method (59) to ensure that no undesired mutations were introduced during the amplification or cloning processes.

Genetic methods and construction of yeast strains. Standard methods were used for culturing and transformation of yeast strains (32, 60). The yeast strains used or constructed are listed in Table 1. Strain H2543 is the same as RY1-1 described by Romano et al. (58). Strain H2544, a kind gift of Patrick R. Romano, was constructed in the same way as H2543 but contains only a single copy of the *GAL-CYC1-PKR* construct integrated at the *LEU2* locus. The control strain J110 was constructed by first modifying the multiple cloning site in the yeast *LEU2* integrating vector pRS305 (62) by removing all sequences between *Xho*I and *Xba*I and then religating. The resultant plasmid, pC390, was then linearized at the unique *Eco*RV site in the *LEU2* gene and used to transform strain H1894, generating J110. Strain J110 is identical to strain H2544 except that the fragment inserted at the *LEU2* locus lacks the *GAL-CYC1-PKR* allele. Strain J46 is isogenic to strain H1895, with the *SUI2* allele replaced by *SUI2-S51A*. The *SUI2-S51A* allele was introduced into strain H1895 on plasmid pC50 digested with *Bgl*II to direct integration to *SUI2*. Derivatives resistant to 0.1% 5-fluoroorotic acid (4) were isolated, and strains carrying the *SUI2-S51A* allele were identified by their inability to complement the 3-aminotriazole (3-AT)-sensitive phenotype of a *gcn2*Δ strain or a *SUI2-S51A* strain. Further testing confirmed that only by introducing plasmids encoding both wild-type *GCN2* and *SUI2* could strain J46 display a 3-AT-resistant phenotype. The chromosomal *GCN2* allele in strains H1515 and H1642 was replaced by an unmarked *gcn2*Δ allele, as described previously (19), to create strains H1894 and H1895, respectively.

For the yeast two-hybrid system (21), *GAL4* fusion constructs were introduced into strain Y190. 3-AT resistance of the transformants was assayed on SD medium supplemented with 0.15 mM adenine and 25 mM 3-AT.

Mutagenesis of K3L and isolation of hyperactive K3L alleles. The K3L expression plasmid pC140 was randomly mutated by amplifying the K3L coding sequence, using low-fidelity PCR (6). The primers used for PCR amplification of K3L restored the authentic Leu codon at the second residue of the K3L protein and abolished the *Nco*I restriction site that had been introduced at the initiator AUG codon of K3L in plasmid pOS13. After digestion with *Sac*I and *Xba*I, PCR products were subcloned back into the pEMBLyex4 vector at the *Sac*I and *Xba*I sites, and a pool of the mutated K3L plasmids was introduced into the yeast strain H2543 (Table 1) carrying two copies of the *GAL-CYC1-PKR* construct. Transformants were picked randomly, patched on SD plates, and replica printed to SGal (SD containing 10% galactose in place of 2% glucose) plates. While many of the transformants failed to grow or grew more poorly on SGal medium than a transformant expressing wild-type K3L from plasmid pC140, plasmids were isolated only from transformants that grew better on SGal medium than a transformant expressing wild-type K3L. From a screen of about 410 independent yeast transformants, we identified four hyperactive K3L alleles that conferred greater inhibition of PKR than did wild-type K3L. Isolated plasmids were reintroduced into strain H2543 to verify that the recovered plasmids conferred the enhanced suppressor activity. The four hyperactive K3L alleles were then subcloned as *Sac*I-*Xba*I fragments to pBluescript to facilitate sequencing. The nucleotide sequence of the entire *Sac*I-*Xba*I fragment from the four plasmids was determined by the dideoxy-chain termination technique (59), and single amino acid changes were identified in three of the mutant alleles.

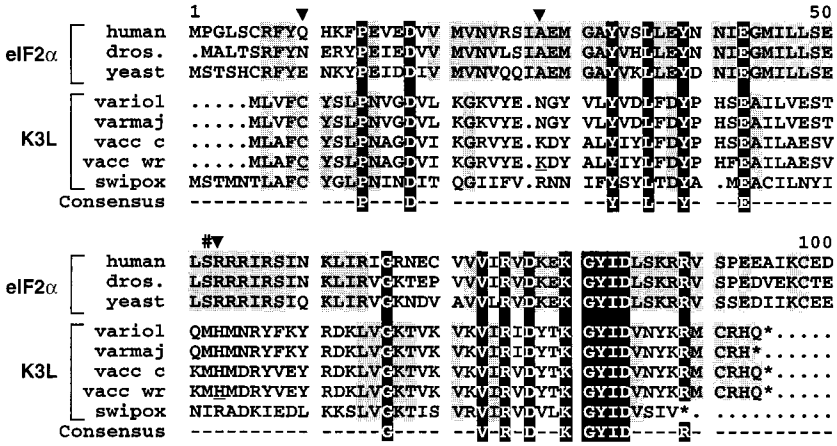


FIG. 1. Alignment of amino acid sequences of eIF2α and viral K3L proteins. The amino acid sequences of the N-terminal third of eIF2α from human (GenBank accession no. J02645), *Drosophila melanogaster* (dros.; L19196), and the yeast *S. cerevisiae* (M25552) are aligned with the full-length amino acid sequences of K3L proteins from variola virus (variol; X69198), variola major virus (varmaj; L22579), vaccinia virus strain Copenhagen (vacc c; M35027), and vaccinia virus strain WR (vacc wr; D00382) and of the C8L protein, the K3L protein homolog from swinepox virus (swipox; L22013). The alignment was generated by using the program PILEUP (Genetics Computer Group, Inc.). The Ser-51 site of phosphorylation in eIF2α is indicated by #. Shown in reverse type and included in the consensus sequence at the bottom of the alignment are residues conserved in all eIF2α and K3L proteins. Shaded are residues conserved among the eIF2α proteins or among the K3L proteins. Black triangles indicate the sites of the hyperactive mutations in the K3L protein, and the amino acid residues which have been altered are underlined.

Immunoblot analysis of PKR and K3L protein expression. Transformants were grown for 2 days in SD medium, and then the cultures were diluted 1:50 into SD medium. After growth for about 10 h, cultures were shifted to synthetic minimal medium containing 10% galactose and 2% raffinose (SGR) and harvested 15 h later. Cells were broken with glass beads in breaking buffer [40 mM piperazine-*N*, *N*′-bis(2-ethanesulfonic acid) (PIPES; pH 6.0), 100 mM NaCl, 2 mM phenylmethylsulfonyl fluoride] by using a Braun homogenizer as described previously (54). Extract samples were fractionated by sodium dodecyl sulfate–4 to 20% gradient polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes, and then the blots were blocked in TBS-T (20 mM Tris-HCl [pH 7.4], 150 mM NaCl, 0.1% Tween 20) containing 5% nonfat dry milk. Immunodetection with anti-PKR monoclonal antibody 71/10 (47), PKR-specific polyclonal antiserum (58) (a gift of Michael G. Katze), and K3L-specific antiserum (2) (a gift of E. Beattie and J. Tartaglia, Virogenetics Corporation) was done at dilutions of 1:1,000, 1:5,000, and 1:1,000, respectively. Subsequent washing and enhanced chemiluminescence (ECL) detection were done as specified by the manufacturer of the ECL kit (Amersham).

IEF gel electrophoresis. Isoelectric focusing (IEF) analysis was conducted as previously described (19), with the following modifications. Yeast strains were grown for 2 days in SD medium, and then the cultures were diluted 1:50 into SD medium. After growth for about 10 h, cultures were shifted to SGR medium and harvested 15 h later. Cells were broken with glass beads in IEF breaking buffer (40 mM PIPES [pH 6.0], 100 mM NaCl, 15 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 50 mM NaF, 35 mM β-glycerophosphate, 10 mM 2-aminopurine). Detection of eIF2α by immunoblot analysis using antiserum specific for yeast eIF2α (11) was carried out by using ECL as described above.

Binding assay of the interaction between PKR and the K3L protein. Yeast strains were grown as described above for the immunoblot analysis, and cells were broken with glass beads in breaking buffer (50 mM Tris-HCl [pH 8.0], 100 mM NaCl, 20% glycerol, 5 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, 1 μM pepstatin A, 0.15 μM aprotinin, 1 μM leupeptin) by using a Braun homogenizer as described previously (54). Extracts containing 300 μg of total protein were incubated with 40 μl of an Ni^{2+}-nitrilotriacetic acid-agarose (Qiagen) suspension in 120 μl of binding buffer (20 mM Tris-HCl [pH 8.0], 100 mM NaCl, 10% glycerol, 5 mM $MgCl_2$, 0.1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 1 μM pepstatin A, 0.15 μM aprotinin, 1 μM leupeptin) for 1 h at 4°C. The beads were pelleted in a microcentrifuge and washed three times with 200 μl of binding buffer. Proteins bound to beads were eluted twice with 300 μl of elution buffer (20 mM Tris-HCl [pH 8.0], 250 mM NaCl, 500 mM imidazole). Fractions of eluents were subjected to SDS-PAGE, immunoblotted, and probed with antiserum specific for PKR or the K3L protein as described above. Antigen-antibody complexes were detected by ECL (Amersham) as described by the manufacturer.

RESULTS

Expression of K3L suppresses the toxicity of PKR in yeast. The K3L protein, an inhibitor of PKR, has significant sequence similarity to the N-terminal one-third of eIF2α, the PKR substrate. Comparison of the amino acid sequences of the K3L gene products from various poxviruses, including vaccinia virus, variola virus, and swinepox virus, reveals high sequence conservation, especially at the C and N termini of the proteins (Fig. 1). When the K3L protein sequences are compared to the sequence of eIF2α, the most striking homology is found near the C terminus of the K3L protein and in a region encompassing residues 72 to 83 of eIF2α (Fig. 1). Comparison of the mammalian, insect, and yeast sequence of eIF2α reveals high conservation throughout the length of these ca. 300-residue proteins; however, of particular note is their perfect identity over 19 residues flanking the site of phosphorylation by the eIF2α kinases at Ser-51 (Fig. 1). Strikingly, the K3L proteins lack similarity to eIF2α in the vicinity of the Ser-51 residue, and the strongest homology occurs about 30 residues away {amino acids 72 to 83 of yeast eIF2α [V(I/L)RVDKEKG YID]}, where up to 10 of 12 residues are perfectly conserved between eIF2α and the K3L protein.

The amino acid sequence conservation between the K3L protein and eIF2α led to the proposal that the K3L protein functions as a pseudosubstrate inhibitor of PKR, and previous results are consistent with this idea. To gain further insights into the mechanism of inhibition of PKR by the K3L protein and to increase our understanding of pseudosubstrate inhibition of protein kinases, we initiated a genetic analysis of K3L. Overexpression of human PKR is toxic in yeast due to the accumulation of high levels of eIF2α phosphorylated on Ser-51 and the ensuing inhibition of general protein synthesis (10, 18). To perform an in vivo mutational analysis of K3L, we first tested whether expression of K3L would suppress the toxicity associated with high-level expression of PKR in yeast. The coding sequence for the K3L gene (strain WR) was subcloned into the yeast expression vector pEMBLyex4 as described in Materials and Methods. In the resulting plasmid (pC140), K3L expression is under the control of a galactose-inducible promoter (*GAL-CYC1*), such that K3L expression is repressed when cells are grown on glucose medium and induced when cells are grown on galactose medium. The K3L expression plasmid was introduced into an isogenic set of strains containing zero, one, or two copies of PKR (strains J110, H2544, and H2543, respectively). Expression of PKR in these strains is also

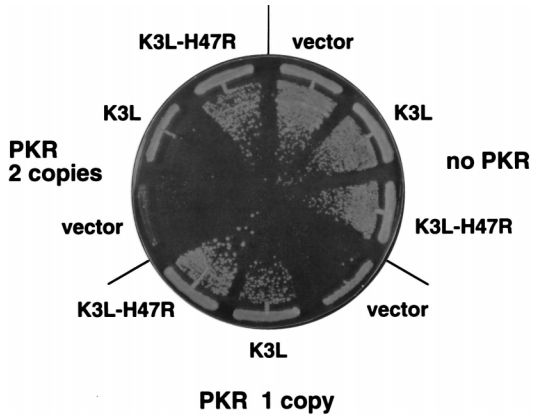


FIG. 2. Coexpression of K3L suppresses the toxicity of PKR expression in yeast. Plasmids expressing wild-type K3L (pC140) or the K3L-H47R allele (pC407) under the control of a yeast *GAL-CYC1* hybrid promoter, or the vector pEMBLyex4 alone, were introduced into the isogenic strains J110, H2544, and H2543, containing, respectively, zero, one, or two copies of a *GAL-CYC1-PKR* construct integrated at the *LEU2* locus. The indicated transformants were streaked on minimal SGal medium supplemented with only the required nutrients, where the expression of both PKR and K3L was induced, and incubated at 30°C for 4 days.

under the control of the chimeric *GAL-CYC1* promoter. As shown in Fig. 2, vector transformants of the yeast strains containing one or two copies of the *GAL-CYC1-PKR* construct exhibited a severe slow-growth phenotype when streaked on galactose medium. Introduction of plasmid pC140 into the strains carrying the PKR expression constructs conferred a substantial (H2544, one copy of PKR) or modest (H2543, two copies of PKR) restoration of growth on galactose medium. High-level expression of K3L had no effect on the growth rate of the strain (J110) lacking PKR (Fig. 2). These results show that expression of K3L can suppress the toxicity of PKR in yeast, and they establish a system with which to perform a mutational analysis on K3L.

Isolation of hyperactive K3L alleles. To gain insights into how PKR recognizes its substrate or pseudosubstrate, we performed a mutational analysis of K3L. As described in Materials and Methods, a pool of randomly mutated K3L genes was screened to identify alleles that were more effective inhibitors of PKR than wild-type K3L. The three hyperactive alleles identified in this screen, K3L-C5G, K3L-K22E, and K3L-H47R, contained nucleotide changes resulting in single amino acid substitutions in the K3L coding sequence at the locations indicated in Fig. 1. Interestingly, the H47R mutation, found in the strongest K3L hyperactive allele, increases the homology between the K3L protein and eIF2α immediately adjacent to the phosphorylation site in eIF2α at Ser-51 (Fig. 1). The enhanced suppressor activity of K3L-H47R is depicted in Fig. 2. The growth rate of a K3L-H47R transformant of the strain H2543, with two copies of PKR, was comparable to that of a vector transformant of the isogenic strain J110 expressing no PKR (Fig. 2). Thus, expression of K3L-H47R almost completely suppressed PKR toxicity.

Importance of the C-terminal conserved sequences of the K3L protein for inhibition of PKR. As indicated previously, the pentapeptide sequence KGYID is perfectly conserved in all eIF2α and K3L proteins (Fig. 1). To determine the importance of this pentapeptide sequence, we examined the effect of C-terminal truncations on K3L protein activity. The codons for M84, R83, K82, Y81, and T73 of K3L or K3L-H47R were replaced with stop codons to make C-terminally truncated proteins (Fig. 3A). The ability of the mutant K3L proteins to inhibit PKR toxicity in strain H2543 was examined by assessing the growth of transformant colonies replica plated to SGal medium (Fig. 3B). For both the K3L and K3L-H47R proteins, truncation of five amino acids from the C terminus had little effect, and the yeast transformants grew as well as transformants expressing full-length K3L protein. Removal of one more residue, creating K3L-(1-82), severely inhibited the activity of the wild-type K3L protein, yet the identically truncated K3L-H47R protein retained PKR inhibitory activity. Truncation after residue 81, deleting a single additional residue, eliminated the activity of both the wild-type K3L and K3L-H47R proteins. Further truncations of the K3L protein C terminus to

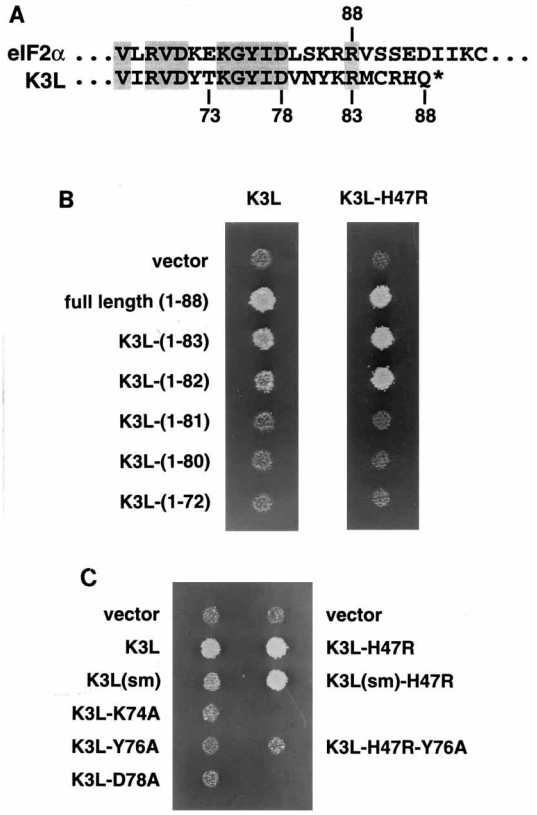


FIG. 3. Mutational analysis of the C-terminal region of the K3L protein, which shows the greatest similarity to eIF2α. (A) Alignment of the amino acid sequences of yeast eIF2α (residues 72 to 97) and the C terminus of the vaccinia virus K3L protein (residues 67 to 88). The shading indicates identical residues in the two proteins. (B) Identification of the C-terminal boundary of the K3L protein required for inhibition of PKR. The K3L or K3L-H47R coding region, as indicated, was truncated by replacing the codons for M84, R83, K82, Y81, and T73 with stop codons. Plasmids expressing the indicated K3L alleles under the control of the *GAL-CYC1* hybrid promoter, or the vector alone, were introduced into the strain H2543, carrying two copies of the *GAL-CYC1-PKR* construct. Patches of transformants were grown to confluence on an SD plate and replica printed to an SGal plate to induce K3L and PKR expression. The plate was incubated at 30°C for 3 (K3L-H47R) or 4 (K3L) days. (C) Effects of point mutations in the C-terminal conserved region of the K3L protein. Point mutations were introduced into the sequence KGYID found near the C terminus of the K3L protein and perfectly conserved in all K3L and eIF2α proteins (Fig. 1). To facilitate the mutagenesis, the K3L(sm) allele, containing translationally silent mutations that introduce restriction sites, was constructed (see Materials and Methods). The K3L-K74A, K3L-Y76A, K3L-D78A, K3L(sm)-H47R, and K3L-H47R-Y76A alleles have the same silent mutations as are present in K3L(sm). Plasmids carrying the indicated K3L alleles were introduced into strain H2543. Patches of transformants were grown to confluence on SD medium and then replica printed to SGal medium, where K3L and PKR expression was induced. The photograph is of a plate incubated at 30°C for 3 days.

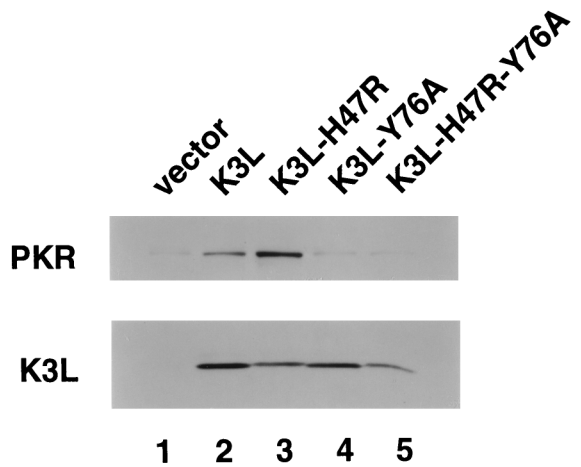


FIG. 4. Immunoblot analysis of PKR and K3L protein levels in yeast strains expressing various K3L mutant proteins. Strain H2543, carrying two copies of the *GAL-CYC1-PKR* construct, was transformed with plasmids carrying the indicated K3L alleles or vector alone. These strains were grown in SGR medium, where PKR and K3L expression is induced, and whole-cell extracts were prepared. Fifteen micrograms of total protein extracts were analyzed by SDS-PAGE on a 4 to 20% gradient gel followed by immunoblot analysis using anti-PKR monoclonal antibodies or polyclonal anti-K3L antiserum, as indicated. Immune complexes were visualized by ECL.

residue 80 or to residue 72, removing the KGYID sequence, similarly result in inactive proteins. These results place the C-terminal boundary of the PKR inhibitory domain of the K3L protein either at the conserved Arg-83 (Fig. 1) or at Lys-82. The importance of Arg-83 for K3L protein activity is particularly intriguing considering that a mutation in the corresponding residue of eIF2α appears to affect phosphorylation by the yeast eIF2α kinase GCN2 (66) (see Discussion).

To test directly the importance of the conserved KGYID sequence for K3L protein function, site-directed mutagenesis was used to make independent substitutions of Ala for Lys-74, Tyr-76, or Asp-78 in the wild-type K3L protein. To facilitate mutagenesis of K3L, translationally silent mutations that introduce restriction sites were engineered in the K3L gene. The K3L(sm) allele, containing the silent mutations, expressed lower levels of the K3L protein than the wild-type gene and conferred a weaker, though still effective, suppression of PKR toxicity (Fig. 3C). All three mutant alleles, K3L-K74A, K3L-Y76A, and K3L-D78A, failed to suppress the toxicity of PKR (Fig. 3C). As a further test of the importance of this sequence motif, the Y76A mutation was introduced into a modified version of the hyperactive K3L-H47R allele containing the same silent mutations. The K3L(sm)-H47R allele remained a potent suppressor of PKR; however, the K3L-H47R-Y76A mutant allele was unable to inhibit PKR toxicity in yeast (Fig. 3C). These results demonstrate that the KGYID sequence in the K3L protein is critically important for the inhibition of PKR, and they suggest that this motif may be important for the recognition of the K3L protein (and, by extension, eIF2α) by PKR.

Expression of K3L alleviates negative autoregulation of PKR expression. To investigate how the mutations that we have identified alter K3L protein function, we first used immunoblot analyses to examine the expression of the K3L and PKR proteins in yeast cells. Whole-cell extracts, prepared from transformants of strain H2543 expressing various K3L proteins, were subjected to SDS-PAGE and immunoblot analysis using either monoclonal PKR antibodies or polyclonal anti-K3L antiserum. As shown in Fig. 4, the hyperactive K3L-H47R protein was expressed at slightly lower levels than wild-type K3L protein (Fig. 4, K3L panel; compare lanes 2 and 3), and the inactive K3L-Y76A and K3L-H47R-Y76A proteins were expressed at levels comparable to those of the corresponding proteins lacking the Ala-76 mutation (compare lanes 4 and 2 and lanes 5 and 3). The fact that the K3L hyperactive allele was expressed at lower levels than the wild-type K3L protein eliminated the possibility that the K3L-H47R hyperactive allele was a more effective inhibitor of PKR simply because the mutation increased the steady-state level of the K3L protein. Similarly, the deleterious effect of the Y76A mutation on K3L function is not due to lowered K3L protein levels. Immunoblot analyses of K3L expression levels in the strain H1894 lacking PKR revealed that the K3L-K74A, K3L-Y76A, and K3L-D78A proteins shown in Fig. 3C were expressed at levels similar to that of the wild-type K3L protein (data not shown); in addition, the K3L-C5G and K3L-K22E hyperactive alleles, like K3L-H47R, were expressed at lower levels than the wild-type K3L protein (data not shown). Finally, the K3L proteins truncated at M84, R83, and K82, which produced functional, partially functional, and nonfunctional proteins, respectively, were expressed at equivalent levels, slightly lower than that of wild-type K3L, in strain H2543 containing PKR (data not shown). These results suggest that the phenotypes associated with various K3L mutations are due to alterations in K3L protein activity rather than changes in K3L protein abundance.

It has been reported that PKR down-regulates its own expression in mammalian cells (1). We and others have shown that PKR also negatively autoregulates its expression in yeast cells (18, 58). Thus, Romano et al. (58) found that the expression levels of various mutant PKR proteins were inversely proportional to their in vivo kinase activities. In addition, the autoregulation of PKR expression is blocked in strains expressing eIF2α-S51A (18, 58). In view of these findings, we reasoned that PKR expression should be inversely related to the effectiveness of K3L inhibition of PKR. As predicted, in yeast cells expressing PKR and an empty vector, PKR expression was low (Fig. 4, lane 1). Expression of wild-type K3L (lane 2) or the hyperactive K3L-H47R allele (lane 3) led to increased PKR levels compared to the strain expressing the empty vector (lane 1), whereas PKR expression was low in strains expressing the inactive K3L-Y76A or K3L-H47R-Y76A protein (lanes 4 and 5). These results demonstrated that the suppression of PKR toxicity in yeast by K3L or the hyperactive K3L-H47R allele was not simply due to lowered PKR expression levels. In addition, they provided biochemical evidence that the K3L and K3L-H47R proteins are effective inhibitors of PKR in yeast, whereas the K3L-Y76A and K3L-H47R-Y76A proteins are functionally defective.

Expression of K3L inhibits phosphorylation of eIF2α by PKR in yeast. Previous work has shown that the K3L protein can inhibit the phosphorylation of eIF2α by PKR in vitro (7, 16). To examine the effects of the mutant and wild-type K3L proteins on eIF2α phosphorylation in yeast, we analyzed eIF2α phosphorylation by using IEF gel electrophoresis. In a *gcn2*Δ strain expressing wild-type eIF2α and the catalytically inactive PKR-K296R kinase, only the basally phosphorylated form of eIF2α was detected (Fig. 5, lane 2). High-level expression of wild-type PKR from the multicopy plasmid p1420 in this strain resulted in almost complete conversion of eIF2α to the form that is phosphorylated on Ser-51 (lane 1). High levels of eIF2α phosphorylation were identified in vector transformants of strain H2544, carrying one chromosomally integrated copy of the *GAL-CYC1-PKR* construct (lane 3). Expression of wild-type K3L (lane 4) or K3L-H47R (lane 5) in strain H2544

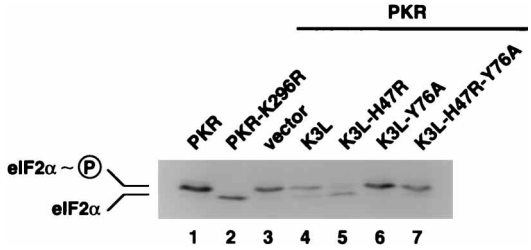


FIG. 5. IEF gel electrophoresis of eIF2α from strains expressing various K3L and PKR proteins. Plasmids expressing either wild-type PKR (p1420; lane 1) or the inactive mutant PKR-K296R (p1421; lane 2) were introduced into the *gcn2*Δ strain H1816; strain H2544, carrying one copy of the *GAL-CYC1-PKR* construct, was transformed with plasmids carrying the indicated K3L alleles or vector alone (lanes 3 to 7). Transformants were grown in SGR medium as described in Materials and Methods to induce PKR and K3L expression. Samples of whole-cell extracts were separated by IEF on a vertical polyacrylamide slab gel as described previously (19), followed by immunoblot analysis using anti-yeast eIF2α antiserum. The immune complexes were detected by ECL. As indicated, the more acidic hyperphosphorylated form of eIF2α, phosphorylated on Ser-51, focuses above the basally phosphorylated species.

significantly lowered the proportion of eIF2α phosphorylated on Ser-51. In fact, the majority (ca. 70%) of the eIF2α protein was not phosphorylated on Ser-51 in the transformants expressing the K3L-H47R protein (lane 5). Finally, high levels of eIF2α phosphorylation were observed in transformants of H2544 expressing the K3L-Y76A or K3L-H47R-Y76A protein (lane 6 or 7). These results established a strong correlation between the ability of the various K3L mutant alleles to suppress PKR toxicity and their ability to inhibit eIF2α phosphorylation in vivo, confirming that the various K3L mutant proteins differed in their abilities to inhibit PKR kinase activity. Based on experiments in mammalian systems (reviewed in reference 65), the levels of eIF2α phosphorylation observed in the strains expressing the K3L-H47R protein would be expected to significantly inhibit protein synthesis. The fact that the cells expressing K3L-H47R were growing well despite the significant level of eIF2α phosphorylation suggests that yeast eIF2B may be less sensitive than mammalian eIF2B to inhibition by phosphorylated eIF2 or that the ratio of eIF2B to eIF2 may be higher in yeast than in mammalian cells.

K3L mutations alter the binding of the K3L protein to PKR. If the K3L protein is a pseudosubstrate inhibitor of PKR, then the simplest model to explain the phenotypes of the various K3L mutations is to propose that they alter the binding of the K3L protein to PKR. To assess the binding of the K3L protein to PKR, we measured the coprecipitation of PKR and K3L proteins expressed in yeast cells. Because the expression of PKR varied depending on the activity of the various K3L alleles (Fig. 4) and because variations in the levels of PKR between different strains would complicate interpretation of the results, we expressed wild-type PKR and the various K3L proteins in a yeast strain containing eIF2α-S51A in place of wild-type eIF2α. As noted above, autoregulation of PKR expression is abolished in yeast strains expressing eIF2α-S51A (18, 58), such that PKR should be produced at high levels independently of which K3L allele is expressed. To facilitate the analysis, wild-type K3L, K3L-H47R, and K3L-Y76A proteins were tagged by the addition of six His residues to the C termini of the proteins. The His-tagged K3L proteins retained the same activities as the untagged proteins when assayed for suppression of PKR toxicity in yeast (data not shown). The three tagged K3L alleles were expressed from the *GAL-CYC1* promoter, along with PKR, in the eIF2α-S51A strain J46. Whole-cell extracts were prepared from the strains grown un-

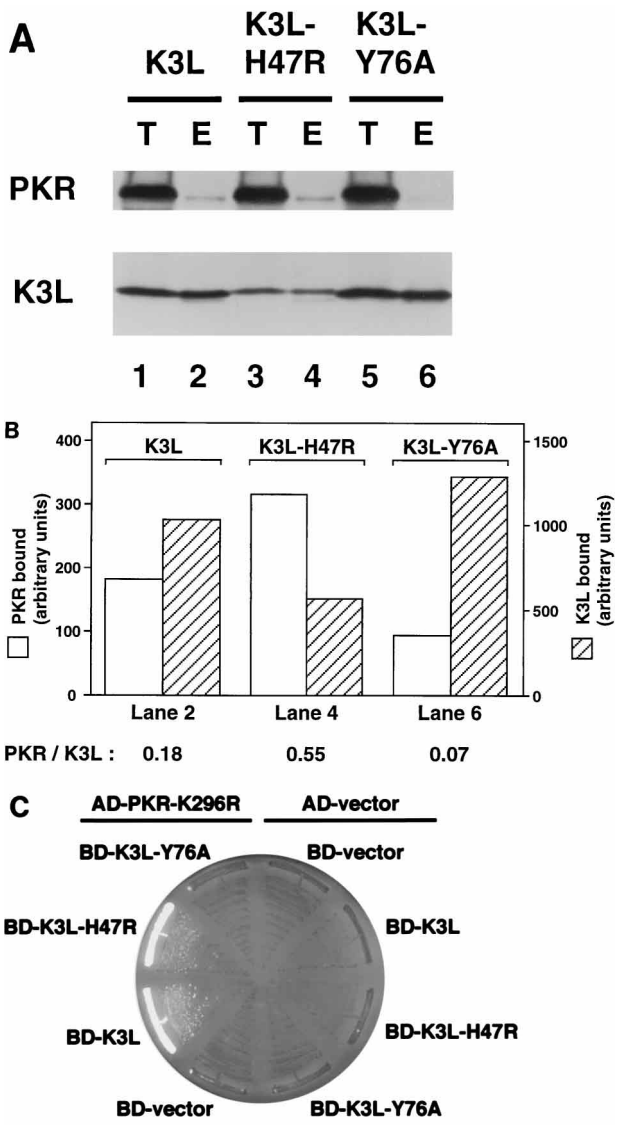


FIG. 6. K3L mutations alter the binding of the K3L protein to PKR. (A) Coprecipitation of PKR with K3L and K3L mutant proteins. Yeast strain J46 (*SUI2-S51A gcn2*Δ) was transformed with plasmids encoding PKR (p1419) and His-tagged wild-type K3L (pC362), K3L-H47R (pC373), or K3L-Y76A (pC374) protein, as indicated. The His-tagged alleles encoded an additional six histidine residues at the C terminus of the K3L protein. Strains were grown under conditions where expression of both K3L and PKR was induced, and whole-cell extracts were prepared and then incubated with Ni^{2+}-agarose beads. The beads were washed, and then the proteins that were specifically eluted with imidazole were separated by SDS-PAGE and immunoblotted with polyclonal anti-PKR or anti-K3L antiserum, as indicated. Immune complexes were visualized by ECL. Lanes 1, 3, and 5, 100 μg of total (T) starting extract; lanes 2, 4, and 6, material precipitated and then specifically eluted (E) from 100 μg of extract. (B) Quantitative densitometry of the results shown in panel A. The amounts of PKR and K3L proteins bound and then eluted from the Ni^{2+}-agarose beads were determined by densitometry of lanes 2, 4, and 6 of the film shown in panel A. The amounts of PKR and K3L proteins recovered are expressed in arbitrary units; the recovery of PKR relative to K3L protein for each sample is listed below the histogram. (C) Yeast two-hybrid analysis of the interaction between PKR and wild-type and mutant K3L proteins. Strain Y190 was cotransformed with plasmids encoding the GAL4 activation domain alone (AD-vector, pGAD424) or a fusion of this *GAL4* domain to PKR-K296R (AD-PKR-K296R) and with plasmids encoding the GAL4 DNA binding domain alone (BD-vector, pGBT9) or a fusion of this binding domain to full-length versions of the wild-type K3L (BD-K3L), K3L-H47R (BD-K3L-H47R), or K3L-Y76A (BD-K3L-Y76A) protein, as indicated. The transformants were streaked on minimal SD medium supplemented with only the required nutrients plus 25 mM 3-AT and incubated at 30°C for 3 days.

der galactose-inducing conditions and subjected to immunoblot analysis to measure the levels of PKR and K3L proteins in each extract. As shown in Fig. 6A (lanes 1, 3, and 5), equal amounts of PKR were expressed in all three strains. Similar to what was observed in Fig. 4, expression of the tagged version of K3L-H47R was lower than that of tagged wild-type or K3L-Y76A protein (compare lanes 1, 3, and 5).

To assess the binding of the three K3L proteins to PKR, the whole-cell extracts were incubated with Ni^{2+}-agarose beads to remove the His-tagged proteins from solution. After extensive washing, the proteins bound to the beads were eluted with buffer containing imidazole and were analyzed by SDS-PAGE and immunoblotting. As shown in Fig. 6A, almost all (92 to 99%) of the K3L proteins in the extracts bound to the Ni^{2+}-agarose beads and eluted with imidazole (compare lanes 2 and 1, 4 and 3, and 6 and 5). Only a small percentage of the total input PKR was precipitated with wild-type K3L protein (compare lanes 1 and 2). This low level of binding between the K3L protein and PKR is consistent with the previously observed interaction between a bacterially expressed glutathione *S*-transferase–K3L fusion protein and PKR synthesized in vitro (14). We reproducibly observed a twofold-greater amount of PKR coprecipitating with the K3L-H47R protein than with wild-type K3L (Fig. 6A and B; compare lanes 4 and 2), whereas less than one-half of the amount of PKR recovered with the wild-type K3L protein was coprecipitated with the K3L-Y76A protein (compare lanes 6 and 2). The results of these binding assays are consistent with the ability of the K3L proteins to suppress the toxicity of PKR in yeast, and they suggest that the H47R mutation in K3L increases the affinity between the K3L protein and PKR, while the Y76A mutation lowers the affinity between the K3L and PKR proteins. Considering that the concentrations of the K3L-H47R and K3L-Y76A proteins in the cell extracts were approximately one-half of and slightly greater than, respectively, that of the wild-type K3L protein (Fig. 6B), the effects of these mutations on the affinity of the K3L protein for PKR are greater than would be calculated from the bound fractions alone. The fact that such a low percentage of PKR was recovered in a complex with the K3L protein suggests that effective inhibition of PKR may require a large molar excess of K3L protein.

To further investigate the interaction between PKR and K3L and K3L mutant proteins, we used the yeast two-hybrid system (21). This assay examines protein-protein interactions by analyzing the ability of *GAL4* fusion proteins to stimulate expression of a *GAL1-HIS3* reporter in vivo. Reconstitution of *GAL4* activity through interaction of the *GAL4* fusion proteins leads to a 3-AT-resistant phenotype in the tester strain Y190. Using this assay, Gale et al. (22) showed that full-length K3L fused to the GAL4 DNA binding domain (BD-K3L) could interact with PKR-K296R fused to the GAL4 activation domain (AD-PKR-K296R) (22). We constructed similar fusions of the GAL4 DNA binding domain with wild-type K3L, K3L-H47R, and K3L-Y76A and then tested their interactions with the AD-PKR-K296R fusion. As shown in Fig. 6C, coexpression of the K3L or K3L-H47R fusion with the PKR-K296R fusion conferred resistance to 3-AT. However, no interaction was detected between the K3L-Y76A fusion and the PKR-K296R fusion. Transformants expressing these latter two fusions grew similarly to transformants containing empty vectors (Fig. 6C). The expression of the three K3L fusion proteins was examined by immunoblot analysis, and as was observed for the intact K3L proteins in Fig. 4, the K3L-Y76A fusion was expressed at the same level as the wild-type K3L fusion and at a slightly higher level than the K3L-H47R fusion (data not shown). Therefore, the failure to detect an interaction between the K3L-Y76A and PKR-K296R fusions was not due to reduced protein levels. These results are consistent with the results of the binding assays (Fig. 6A and B), and they provide further support for the model that sequences near the C terminus of the K3L protein, including the KGYID sequence conserved in eIF2α, are important for the recognition and binding of the K3L protein by PKR.

Allele-specific inhibition by K3L of the yeast eIF2α kinase GCN2. The results presented thus far support the model that the K3L protein functions as a pseudosubstrate inhibitor of PKR. Consistent with this idea, it was shown previously that the K3L protein will also inhibit mammalian HRI in vitro (7). As a further test of the model that the K3L protein is a pseudosubstrate inhibitor of the eIF2α kinases, we examined the ability of the K3L gene product to inhibit the yeast eIF2α kinase GCN2. GCN2 functions in the general control of amino acid biosynthesis in yeast, and phosphorylation of eIF2α by GCN2 is required for yeast cells to grow under conditions of amino acid starvation. We found that high-level expression of K3L impaired the growth of yeast cells under histidine starvation conditions imposed by the histidine analog 3-AT (data not shown). While this report was in preparation, Qian et al. (56) published similar results showing that the K3L protein can inhibit the kinase activity of GCN2 both in vivo and in vitro.

GCN2 is normally activated in yeast cells only under starvation conditions; however, mutant alleles of *GCN2* that elicit activation of the kinase in the absence of starvation have been isolated (57). The strongest of these constitutively activated *GCN2^c* alleles are pleiotropic and lead to a slow-growth phenotype due to extensive eIF2α phosphorylation and inhibition of general protein synthesis (57). This phenotype resembles the toxic effects associated with expressing PKR in yeast. To characterize in greater detail the inhibition of GCN2 by K3L, we examined the ability of K3L to suppress the slow-growth phenotypes associated with the *GCN2^c-513* and *GCN2^c-516* alleles. The galactose-inducible K3L expression vector pC140 or an empty vector was introduced into an isogenic set of strains expressing either wild-type GCN2, GCN2^c-513, or GCN2^c-516. The growth rate of the transformants on SD medium, where K3L is not expressed, reflects the severity of the *GCN2^c* alleles. Strains expressing the GCN2^c-516 protein showed a partial slow-growth phenotype compared to strains expressing wild-type GCN2, while the strains containing the GCN2^c-513 protein showed a more severe slow-growth phenotype on SD medium (Fig. 7A). When K3L expression was induced in these transformants by streaking on galactose medium, we observed that K3L conferred a strong suppression of the slow-growth phenotype of the *GCN2^c-513* strain but had only a modest effect on growth of the *GCN2^c-516* strain (Fig. 7B). It is striking that the *GCN2^c-513* strain expressing K3L grew better than the *GCN2^c-516* strain expressing K3L, while in the absence of K3L, the relative growth rates of the two strains were reversed (Fig. 7B). These results indicate that the K3L protein is a more effective inhibitor of GCN2^c-513 than of GCN2^c-516 even though GCN2^c-513 is the more highly activated of the two kinases. In genetic analyses, allele-specific interactions are often indicative of direct protein-protein interactions, and this interpretation would be consistent with the idea that the K3L protein directly binds GCN2 and reduces its activity by acting as a pseudosubstrate inhibitor.

To characterize further the allele specificity in the inhibition of the GCN2^c proteins by K3L, we examined additional *GCN2^c* alleles with various combinations of point mutations. Expression vectors encoding wild-type K3L, the hyperactive K3L-H47R allele, or no K3L were introduced into an isogenic series of strains differing only in the *GCN2* alleles that they con-

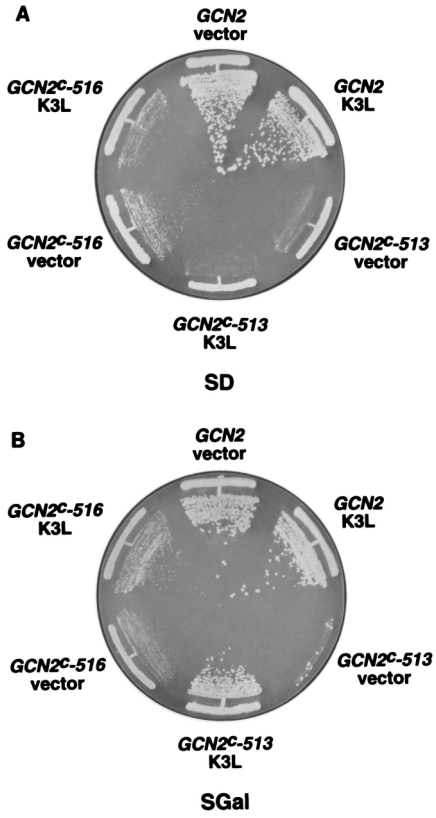
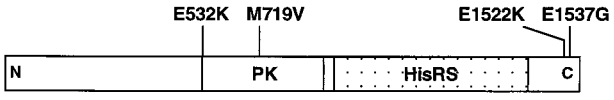


FIG. 7. Expression of K3L suppresses the slow-growth phenotype of strains containing GCN2^c kinases. The isogenic strains H1402 (*GCN2*), H1608 (*GCN2^c-M719V-E1537G* [*GCN2^c-513*]) and H1613 (*GCN2^c-E532K-E1522K* [*GCN2^c-516*]) were transformed with the K3L expression plasmid pC140 or with the vector pEMBLyex4 alone. The transformants were streaked on minimal SD (A) or SGal (B) medium supplemented with only the required nutrients and incubated at 30°C for 2 days (A) or 4 days (B).

tained, and the growth of the resulting strains was examined by streaking on galactose medium. Included in the results of this experiment shown in Fig. 8 are the data from Fig. 7B, where we found that expression of K3L was a more potent inhibitor of the slow-growth phenotype of *GCN2^c-513* than of *GCN2^c-516*. These two *GCN2^c* alleles contain different point mutations both in the protein kinase domain and near the C terminus of the protein. In each case, both mutations must be present to observe the slow-growth phenotypes characteristic of *GCN2^c-513* and *GCN2^c-516* (57). To identify which of the mutations resulted in the heightened sensitivity to K3L, we examined the ability of K3L to suppress the slow-growth phenotypes of strains expressing other GCN2^c kinases containing different combinations of activating mutations. As shown in Fig. 8, the *GCN2^c* alleles in strains H1608 and H1685 that contain the *M719V* mutation in the protein kinase domain were effectively suppressed by expression of K3L, whereas the *GCN2^c* alleles containing the *E532K* mutation in the kinase domain, or the *E1522K* or *E1537G* mutation near the C terminus, were relatively insensitive to expression of K3L. Because the *GCN2^c* allele containing only the *M719V* mutation does not confer a growth defect, we were unable to assess its relative sensitivity to K3L expression (data not shown). Interestingly, the *GCN2^c* allele in strain H1609 was insensitive to expression of wild-type K3L but was effectively suppressed by expression of the hyperactive K3L-H47R allele. This increased sensitivity to the K3L-H47R allele, which was also observed for PKR, suggests that the triple mutation in the *GCN2^c* allele in H1609 increases the structural similarity between the kinase domains of GCN2 and PKR. Finally, the *M719V* mutation in *GCN2* is in a region of the kinase domain that is highly conserved among the eIF2α kinases (10, 57). This mutation is located within the hexapeptide sequence LFIQME, which is perfectly conserved between GCN2 and PKR and is found to the N-terminal side of kinase subdomain V and adjacent to a large insert region in the kinase. The conservation of this sequence among the eIF2α kinases has led to the proposal that it may function in substrate recognition; however, mutations in this region were not found to alter the binding interaction between the K3L protein and PKR (14). Our findings demonstrate that mutations in this region of GCN2 can alter the sensitivity to a pseudosubstrate inhibitor, and it will be interesting to determine if mutations in this region of the kinase domain also affect substrate recognition.

DISCUSSION

Mutational analysis of K3L supports the model that the K3L protein is a pseudosubstrate inhibitor of PKR. Identification of the sequence homology between the K3L protein and eIF2α, and the observation that vaccinia virus mutants lacking K3L show a greater sensitivity to interferon, led to the proposal that the K3L protein was an inhibitor of PKR (3). Subsequently, it was shown that the K3L protein inhibits phosphorylation of eIF2α by PKR both in vivo (2, 15, 16) and in vitro (7, 16, 34), and both biochemical (7, 14, 34) and yeast two-hybrid (13, 14, 22) assays demonstrated a direct interaction between



strain	*GCN2* allele	growth		
		vector	K3L	K3L-H47R
H1402	*wild type*	++++	++++	++++
H1414	*E532K*	+++	+++	+++
H1608 (*GCN2^c-513*)	*M719V E1537G*	+/-	+++	+++
H1613 (*GCN2^c-516*)	*E532K E1522K*	+	+*	+*
H1685	*M719V E1522K*	+	+++	+++
H1692	*E1537G*	+	+*	++
H1611	*E532K E1537G*	+/-	+	++
H1609	*R699W D918G E1537G*	+/-	+/-	+++

FIG. 8. Allele-specific suppression by K3L of the slow-growth phenotype associated with GCN2^c kinases. Isogenic yeast strains expressing wild-type *GCN2* (H1402) or *GCN2^c* alleles with the indicated amino acid substitutions were transformed with the wild-type K3L expression plasmid (pC140), the K3L-H47R expression plasmid (pC407), or the vector pEMBLyex4 alone, as indicated. The transformants were streaked on SGal medium to induce K3L expression and incubated at 30°C for 4 days. The relative growth rates are summarized quantitatively by the number of plus signs. Growth indicated by +* is slightly better than growth indicated by +. The upper panel is a schematic of the GCN2 protein with the protein kinase domain (PK) and the histidyl-tRNA synthetase-related region (HisRS) indicated by shading. The locations of the activating mutations in the GCN2^c kinases used in these studies are indicated above the coding region.

the K3L protein and PKR. These data combined with the ability of eIF2 to compete with K3L protein for binding to PKR (34) led to the model that the K3L protein is a pseudosubstrate inhibitor of PKR. Our data demonstrating that mutations in the residues conserved between the K3L protein and eIF2α are required for inhibition of PKR by K3L and that the ability of various K3L alleles to inhibit PKR in vivo correlates with their binding affinity to PKR in vitro provide new evidence that the K3L protein functions as a pseudosubstrate inhibitor of PKR.

Among the best-characterized examples of pseudosubstrate inhibitors are PKI, the heat-stable protein inhibitor of PKA, and the regulatory domains in protein kinase C (PKC) and the twitchin kinase (a member of the myosin light-chain kinase family) (reviewed in reference 38). These regulatory domains have sequence similarity with authentic substrate phosphorylation sites except that the serine phospho-acceptor residue is replaced by a nonphosphorylatable amino acid (in these examples, Ala replaces Ser). In these and other examples of pseudosubstrate inhibition, the sequence similarity between the pseudosubstrate inhibitor and authentic substrates is restricted to the residues immediately surrounding the site of phosphorylation in the substrate. Further supporting the importance of local sequence elements for pseudosubstrate inhibition of protein kinases are experiments showing that introduction of Ala at the phospho-acceptor site converted PKA synthetic peptide substrates into inhibitors (37). Surprisingly, the K3L protein has very little sequence similarity to the sequence of eIF2α flanking the phosphorylation site at Ser-51. As discussed below, this feature of the K3L protein suggests that the eIF2α kinases recognize substrates at least partly through interactions with sequences a considerable distance from the phosphorylation site.

Mutational analysis of PKI and peptides derived from the pseudosubstrate regions of PKC and myosin light-chain kinase have revealed the importance of the similarity between these inhibitors and authentic substrates. Inspection of protein kinase PKA phosphorylation sites has led to the identification of a preferred phosphorylation site motif of RRxS (39, 42). Point mutations in PKI that alter the basic residues at the P-2 and P-3 positions severely impair the PKA inhibitory activity (67). Similarly, the inhibitory activity of pseudosubstrate inhibitory peptides derived from PKC are critically dependent on Arg at the P-3 position, consistent with the identification of the xRxx SxRx consensus sequence for PKC phosphorylation sites (41). No similar homologies can be identified between the K3L protein and the residues flanking Ser-51 in eIF2α. However, the hyperactive K3L-H47R variant identified in this report has increased homology with eIF2α. The H47R mutation in the K3L protein aligns with the R52 residue in eIF2α immediately adjacent to the site of phosphorylation in eIF2α. This result may suggest that the eIF2 kinases recognize the sequences flanking Ser-51 in eIF2α. In addition, the fact that the K3L-H47R protein bound more tightly to PKR than the wild-type K3L protein demonstrates that these residues near the site of phosphorylation can contribute to the binding affinity between PKR and a substrate or pseudosubstrate.

The finding that the H47R mutation in K3L increases K3L inhibitory activity provides a model to explain the activity of the swinepox C8L protein, a K3L homolog from this virus (Fig. 1). Our truncation analysis on the wild-type K3L protein demonstrated a strong requirement for R83 (Fig. 2). However, when the K3L-H47R protein was truncated at R83, it retained activity. The swinepox C8L protein is truncated at its C terminus relative to the vaccinia virus K3L protein and lacks the residue corresponding to R83 (Fig. 1). However, the swinepox protein has an Arg at the position corresponding to H47 in the vaccinia virus K3L protein. We predict that this natural substitution in the swinepox K3L homolog may be critical for its ability to inhibit porcine PKR during viral infections.

Recently, it has been shown that the *mei3*$^+$ gene in *Schizosaccharomyces pombe* encodes a pseudosubstrate inhibitor of the Ran1/Pat1 protein kinase (49). A sequence element in Mei3 resembles two sequences found in the Ran1/Pat1 substrate Ste11, with the Ser or Thr phosphorylation site residues replaced by Arg-81 in Mei3. Li and McLeod (49) found that when they substituted Ser for Arg-81, the Mei3-R81S protein was converted into an efficient substrate of Ran1/Pat1. We performed similar experiments with the K3L gene product; however, we were unable to demonstrate phosphorylation of the K3L protein by PKR. First, the seven residues (48 to 54) of eIF2α surrounding the phosphorylation site at Ser-51 were substituted for the corresponding residues in the K3L protein, as aligned in Fig. 1. A similar chimera with the Ser-51 residue replaced by Ala was also constructed. These chimeric constructs were expressed in yeast cells in the presence or absence of PKR. Neither construct was able to suppress PKR toxicity in yeast; however, we were also unable to detect any K3L proteins expressed from these constructs (data not shown). This lack of detection may be the result of protein instability or the loss of an epitope; however, it prevents us from drawing any strong conclusions from these experiments. We additionally made chimeras by combining the N-terminal portion of eIF2α or eIF2α-S51A (residues 1 to 77) with the C-terminal 17 residues of the vaccinia virus K3L protein. These constructs were unable to suppress PKR toxicity in yeast; however, we were again unable to detect the proteins (data not shown). Chimeras that encoded the N-terminal portion of the K3L or K3L-H47R protein (residues 1 to 66) replacing residues 1 to 77 in full-length eIF2α were also constructed. These chimeric proteins were expressed well in yeast; however, they were unable to suppress PKR toxicity or substitute for the essential function of eIF2α in yeast (data not shown). These latter results with the full-length K3L-eIF2α chimeras suggest that the small size of the K3L protein is important for its inhibitory activity and that residues in the N-terminal third of eIF2α play important roles in eIF2α function and are not simply necessary for phosphorylation of eIF2α and regulation of translation.

The ability to coprecipitate PKR and K3L proteins from yeast cells, as well as the positive interactions observed in the yeast two-hybrid assays, are consistent with the model that the K3L protein is a pseudosubstrate inhibitor that blocks PKR function by binding to the kinase active site. In addition, the finding that the K3L-H47R protein is more effective than the wild-type K3L protein in suppressing PKR toxicity in yeast and binds better to PKR than does the wild-type K3L protein further supports the idea that the K3L protein is a competitive inhibitor of PKR. Previous studies using yeast two-hybrid assays have localized a K3L-interacting region in the C-terminal portion of the PKR kinase domain (14, 22). One surprising aspect of our studies is the relatively poor binding between PKR and the K3L protein. Only a small percentage of the total PKR was coprecipitated with the K3L protein, or even the hyperactive K3L-H47R protein, suggesting a rather weak interaction between the K3L and PKR proteins. A similar low percentage of complex formation was observed in a previous study examining the interaction between a bacterially expressed glutathione *S*-transferase–K3L fusion protein and PKR expressed in a rabbit reticulocyte lysate (14). One prediction of these results is that the K3L protein should be expressed to higher levels than eIF2α in the yeast cells in which K3L is suppressing PKR toxicity and blocking eIF2α phosphorylation. Using the K3L-eIF2α fusion protein described above

as a standard to compare our anti-K3L and anti-eIF2α antisera, we estimate that wild-type K3L protein was expressed at roughly fourfold-higher levels than eIF2α in our yeast strains (data not shown). We were unable to estimate the relative levels of K3L protein and PKR in yeast; however, based on the inefficient complex formation between PKR and K3L proteins, we suspect that the K3L protein must be expressed at higher levels than PKR to achieve the extensive inhibition of PKR function observed in these studies. An alternative model to account for the efficient inhibition of PKR despite the unstable interaction between PKR and the K3L protein observed in the binding assays is that the off rate of the K3L · PKR complex is high. As long as the on rate for K3L protein binding to PKR is greater than the on rate for eIF2 binding, the kinase would be effectively inhibited. In support of this idea, we can detect in our binding assays an interaction between PKR and the K3L protein, but not eIF2 (data not shown), suggesting that the K_D for the eIF2 · PKR complex is greater than the K_D for the K3L · PKR complex.

Evidence that substrate recognition by the eIF2 kinases involves sequence elements conserved in the K3L protein and distant from the site of phosphorylation in eIF2α. In in vitro assays, the PKR and HRI kinases were previously shown to phosphorylate peptides rich in basic residues (55). Examination of the sites phosphorylated in these peptides suggested that both kinases preferred to phosphorylate Ser or Thr residues followed by Arg residues. This finding is consistent with the sequence flanking the Ser-51 site in eIF2α where three Arg residues immediately follow Ser-51 ($SELS_{51}RRR$). In addition, a synthetic peptide corresponding to eIF2α residues 45 to 56 was a substrate for both PKR and HRI in vitro (52). However, in these experiments, the peptides were rather poor substrates for the eIF2 kinases. The PKR and HRI kinases were found to phosphorylate eIF2 with a K_m of around 1 μM, while phosphorylation of the synthetic peptide corresponding to eIF2α residues 45 to 56 had a K_m of around 1 mM (52). This 3-orders-of-magnitude difference in the efficiency of phosphorylating a protein versus a model peptide substrate by the eIF2 kinases is in contrast to the similar efficiencies observed for protein and model peptide substrates of PKA (40).

One explanation for the low efficiency of phosphorylation of the short model peptides by PKR and HRI is that these kinases utilize additional elements in eIF2α for substrate recognition. Previously, it has been shown that denatured eIF2α is a rather poor substrate for phosphorylation by the eIF2α kinases (45). This finding suggests that the kinase recognition site of eIF2α requires the proper secondary or tertiary structural arrangement of sequence elements. Our finding that sequences near the C terminus of the K3L protein are important for the interaction between the K3L protein and PKR suggests that the corresponding conserved sequences in eIF2α may be important for kinase-substrate recognition. Consistent with this idea is the finding that an R88C mutation in yeast eIF2α can suppress the toxic effects of a hyperactive $GCN2^c$ kinase. IEF polyacrylamide gel electrophoresis analysis showed that the R88C mutation impaired phosphorylation of eIF2α by the $GCN2^c$ kinase (66). In this current analysis, we found that truncation of the K3L protein to the corresponding residue (Arg-83) abolished K3L function. These results suggest that Arg-88 may be part of the eIF2α kinase recognition motif. In addition, we have found that point mutations in the conserved KGYID sequence of the K3L protein impair both the binding of the K3L protein to PKR and the ability of the K3L protein to inhibit PKR kinase activity. From these results, we propose that the KGYID sequence, perfectly conserved in all eIF2α and K3L proteins and located over 30 residues from the phosphorylation site at Ser-51, forms part of the eIF2α kinase recognition motif. Consistent with this idea, we have identified mutations in the KGYID sequence in eIF2α that block translational regulation and reduce phosphorylation of eIF2α on Ser-51 in yeast strains expressing the GCN2 kinase (49a).

The identification of sequence motifs distant from the site of phosphorylation that affect kinase recognition is not unprecedented. The best-characterized examples of protein kinases requiring sequences distant from the phosphorylation site for substrate recognition are the Jun kinases (JNKs). Efficient phosphorylation of the transcription factor c-Jun at Ser-63 and Ser-73 by the JNKs requires sequences located between residues 30 and 60 in c-Jun (17, 29, 35). This region of c-Jun is referred to as a docking site for the JNKs, and it has been shown that the JNKs will bind to this region of Jun proteins even if the phosphorylation sites are absent (35). It is proposed that the docking site enhances phosphorylation of c-Jun by increasing the local concentration of JNKs near its substrate. Residues around the phosphorylation sites at Ser-63 and Ser-73 in c-Jun have also been found to be important for efficient phosphorylation of c-Jun by the JNKs (35). We propose that the KGYID sequence element in eIF2α functions similarly to the docking site in c-Jun. Recognition of the KGYID sequence may increase the concentration of the eIF2α kinases in the vicinity of eIF2α, and then these kinases can phosphorylate the Ser-51 residue in the proper local sequence context. Alternatively, the tertiary structure of eIF2α may place the KGYID sequence adjacent to Ser-51 and enable the kinase to interact with both sequence elements simultaneously. The solution structure of a repeated domain found in the ribosomal protein S1 from *E. coli* and proposed to be present in the eIF2α and K3L proteins (5) suggests that the KGYID sequence and the Ser-51 residue are located on an exposed surface on the same face of the eIF2α protein. We have found that mutations in the residues flanking Ser-51 as well as in the sequence KGYID can affect eIF2α phosphorylation in yeast strains expressing the GCN2 kinase, supporting the importance of these two sequence elements for kinase recognition of eIF2α (49a). Finally, the utilization of residues both near and distant from the site of phosphorylation by the eIF2α kinases and the JNKs is likely to underlie the strong substrate specificities noted for these kinases.

The insights obtained in these studies on the mechanism of substrate recognition by the eIF2α family of protein kinases should help us identify additional substrates for this family of kinases. Based on the observed growth control (44) and cell signalling properties (69) of PKR, it has been proposed that PKR may have additional substrates, including IκB (46, 50). Recently, we found that the PKR kinase has dual specificity and can autophosphorylate as well as phosphorylate substrates on Ser, Thr, and Tyr residues (49b). The definition of the eIF2α kinase substrate recognition motifs will be very helpful as we search sequence databases for potential Ser/Thr and Tyr substrates of this family of protein kinases. The identification of allele-specific interactions between mutations in the kinase domain of the $GCN2^c$ alleles and K3L supports the model that the K3L protein directly interacts with the protein kinase domain of the eIF2α kinases. In addition, this result raises the interesting possibility of using K3L to genetically map the substrate binding domain in the eIF2α kinases. Finally, in this study we have demonstrated the usefulness of yeast as a system to study translational regulation by the mammalian PKR kinase, and we have shown that this system can be used to characterize viral inhibitors of the eIF2α kinases. Recently, using this system, Gale et al. (23) have determined that the hepatitis C virus NS5A protein is an inhibitor of PKR. We

anticipate that this system will prove useful for identifying and characterizing additional cellular and viral regulators of the eIF2α kinases, which perform important roles in stress response, growth control, and signal transduction pathways in eukaryotic cells.

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