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Short-sequence paper

Cloning and characterization of an eukaryotic initiation factor-2α kinase from the silkworm, *Bombyx mori*☆

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Abstract

Eukaryotic initiation factor 2α (eIF-2α) kinases are involved in the translational regulations that occur in response to various types of environmental stress, and play an important role in the cellular defense system operating under unfavorable conditions. The identification of additional eIF-2α kinases and the elucidation of their functions are necessary to understand how different eIF-2α kinases can specifically respond to distinct stimuli. Here, we report a novel eIF-2α kinase, termed BeK, from the silkworm, *Bombyx mori*. This gene encodes 579 amino acids and contains all 11 catalytic domains of protein–serine/threonine kinases. Most notably, it contains an ''Ile-Gln-Met-Xaa-Xaa-Cys'' motif, which is highly conserved from yeast to mammalian eIF-2α kinases. BeK does not show any significant homology in the NH_2 terminal regulatory domain, suggesting a distinct regulatory mechanism of this novel eIF-2α kinase. BeK is ubiquitously expressed in the various tissues throughout the final larval stage. Importantly, BeK is activated in *Drosophila* Schneider cells following heat shock and osmotic stress, and activated-BeK has been shown to phosphorylate an eIF-2α subunit at the Ser^{50} site. However, other forms of stress, such as immune stress, endoplasmic reticulum stress and oxidative stress, cannot significantly elicit BeK activity. Interestingly, the baculovirus gene product, PK2, can inhibit BeK enzymatic activity, suggesting that BeK may be an endogenous target for a viral gene product. Taken together, these data indicate that BeK is a novel eIF-2α kinase involved in the stress response in *B. mori.*

Keywords: *Bombyx mori*; Eukaryotic initiation factor 2α kinase; Stress response; Translational regulation

Eukaryotic cells use a translational control system as a rapid response to various types of stress, such as amino acid starvation, heat shock, viral infection, heme deprivation and endoplasmic reticulum stress [1–8]. Under these conditions, eIF-2α kinase phosphorylates the α subunit of eIF 2. The phosphorylated form of eIF-2α sequesters eIF-2B, which is necessary for the exchange of GTP to GDP in the recycling of eIF-2 [9]. Because eIF-2B is a quantitatively limiting factor, phosphorylated eIF-2α is sufficient to sequester all available eIF-2B and stop the initiation of translation [9]. Among the eIF-2α kinase family members, four eIF-2α kinases have been extensively studied. They are heme-regulated eIF-2α kinase (HRI), double-stranded RNA-dependent eIF-2α kinase (PKR), yeast GCN2 protein kinase and endoplasmic reticulum resident kinase (PERK or PEK). HRI is activated under conditions of heme deficiency of reticulocytes, and inhibits protein synthesis [10,11]. PKR is induced by interferons and is activated by low concentrations of double-stranded RNA produced during viral infections [12,13]. Yeast GCN2 kinase is activated by amino acid starvation, and generates a signal that leads to the enhanced expression of genes involved in amino acid biosynthesis [14,15]. PERK is a type I transmembrane protein and is activated in response to a stress signal from the endoplasmic reticulum [7,8]. All these kinases use eIF-2α as a common

Abbreviations: eIF-2α, α subunit of eukaryotic initiation factor; BeK, *Bombyx mori* eIF-2α kinase; HRI, heme-regulated eIF-2α kinase; PKR, double-stranded RNA-dependent eIF-2α kinase; PERK/PEK, endoplasmic reticulum resident kinase; BmNPV; *Bombyx mori* nuclear polyhedrosis virus; AcMNPV, *Autographa californica* multiple nuclear polyhedrosis virus

☆ The nucleotide sequences in this paper have been submitted to the DDJB/EMBL/GenBank databases under the accession number U87236.

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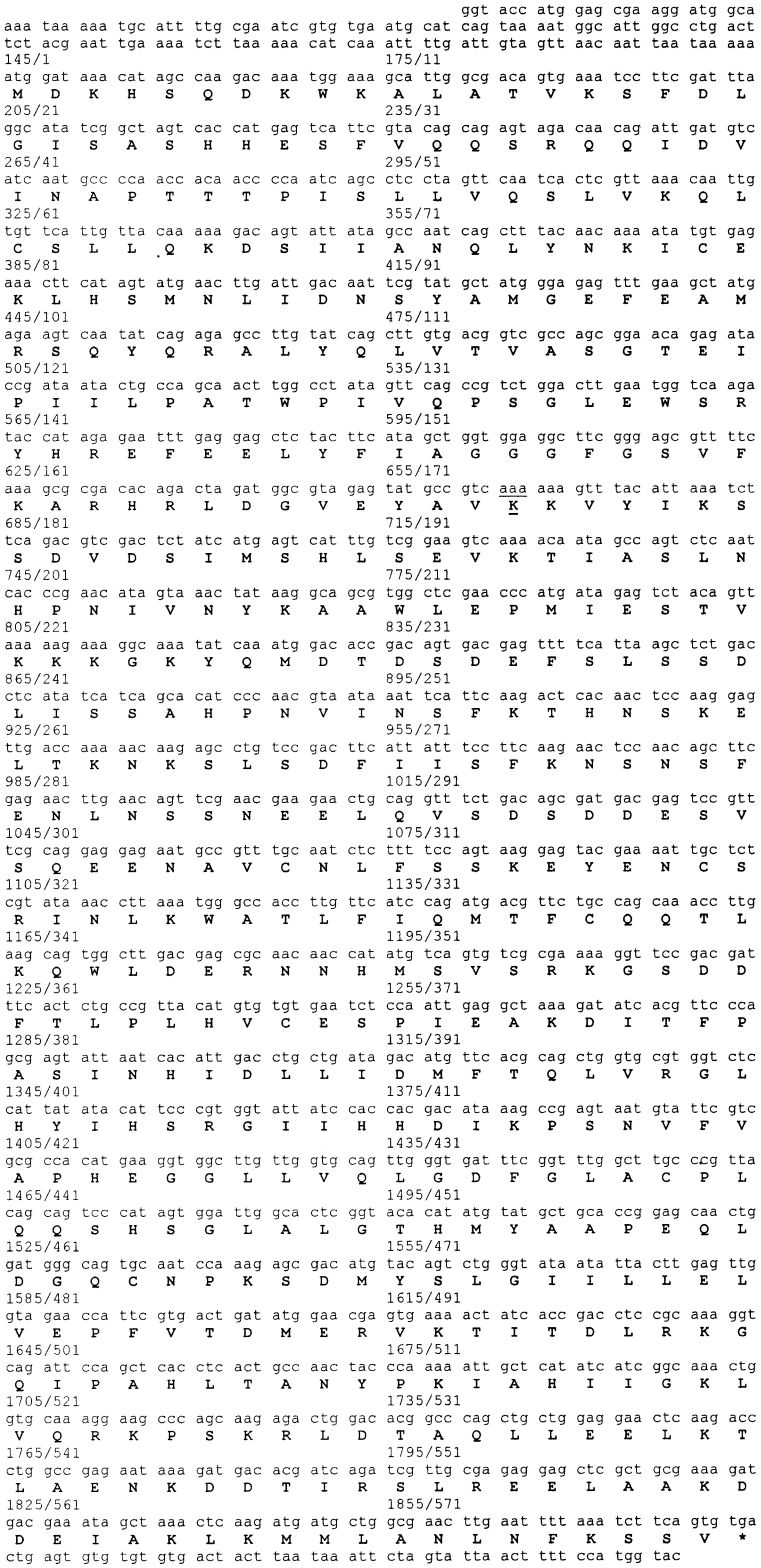
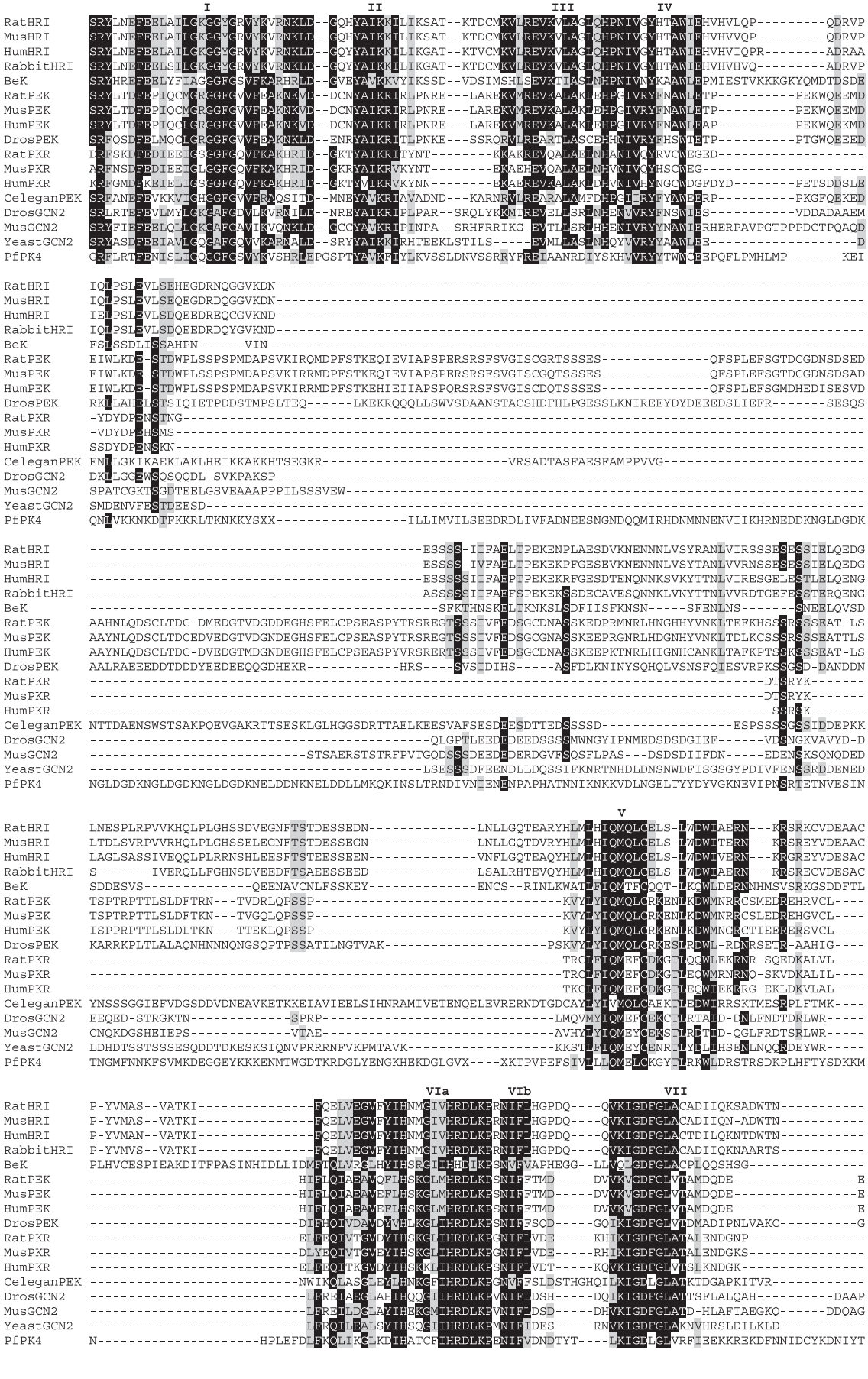


Fig. 1. The nucleotide sequence of the cDNA encoding for BeK and its deduced amino acid sequence. Numbers of the sequence show the position of nucleotides and amino acids, respectively. Asterisk indicates stop codon. The 174th amino acid (Lys^{174}) used to generate catalytically inactive mutant form of BeK is underlined.

A


I
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VIb
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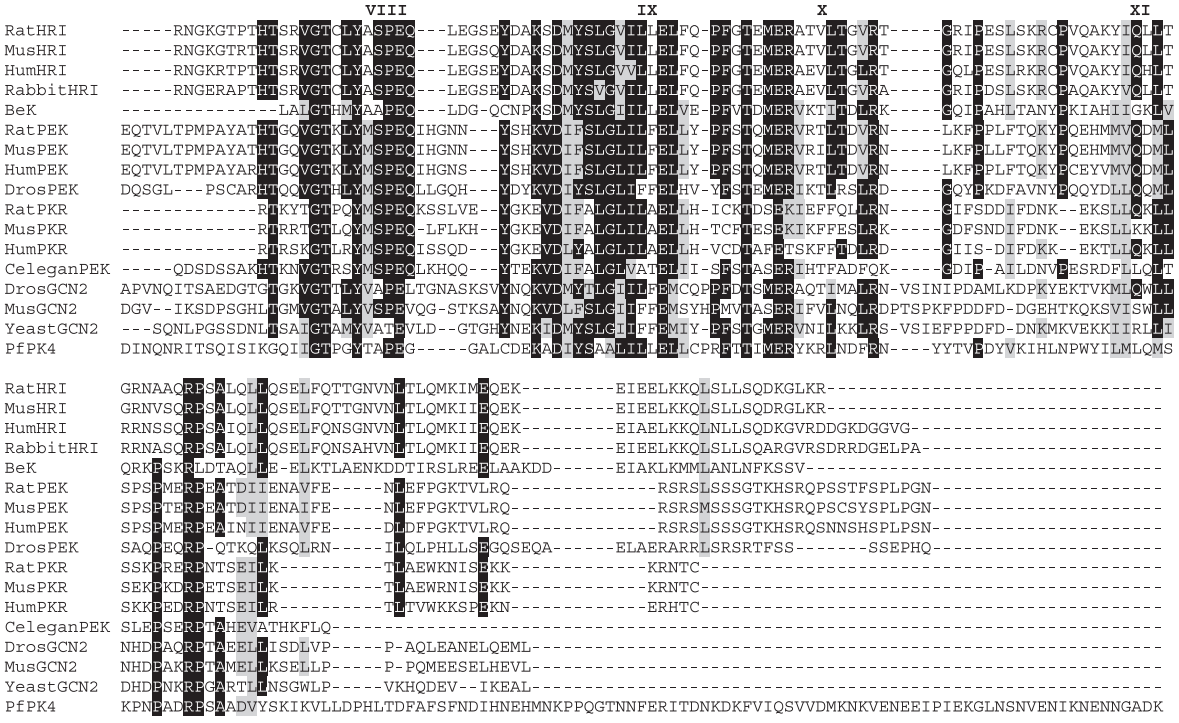

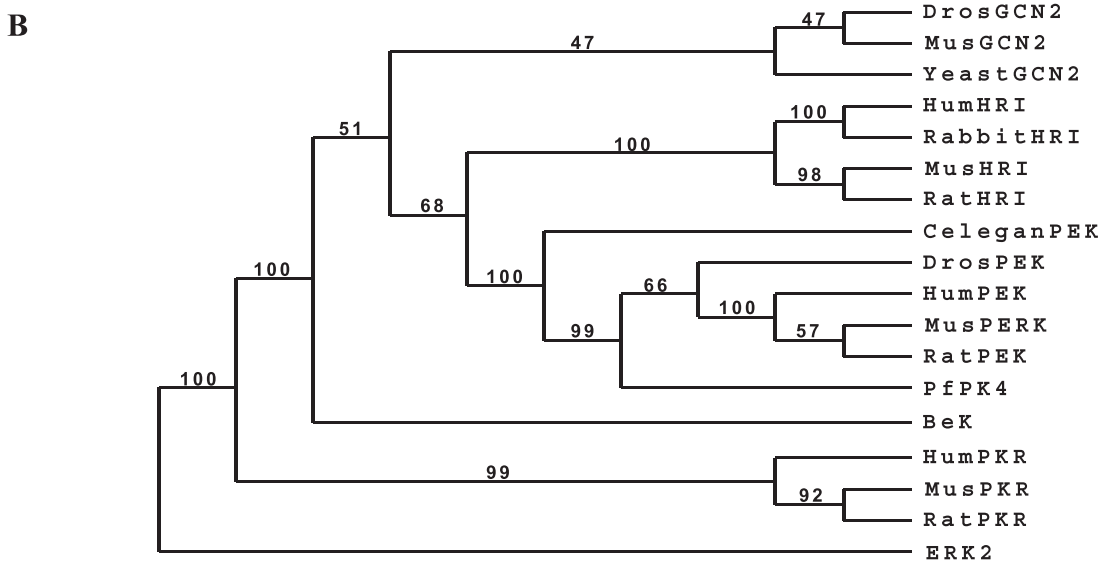

Fig. 2. (A) Multiple alignment of the kinase catalytic domain of BeK to other eIF-2a kinases. The conserved subdomains characteristic of the protein kinases are shown by roman numerals. Identical and similar amino acid residues are highlighted by closed boxes and gray boxes, respectively. Alignment is optimized by introducing gaps using CLUSTAL W program. The long stretches of nonoverlapping sequences of PfPK4 were removed to optimize the multiple alignments, which is indicated by //. RatHRI, rat heme-regulated eIF-2α kinase (HRI) (NP_037355 [23]); MusHRI, mouse HRI (NP_038585 [24]); HumHRI, human HRI (NP_055228 [25]); RabbiHRI, rabbit HRI (P33279 [10]); RatPEK, rat endoplasmic reticulum resident kinase (PEK) (T17455 [7]); MusPEK, mouse PEK (T14351 [8]); HumPEK, human PEK (NP_004827 [26]); RatPKR, rat double-stranded RNA-dependent eIF-2α kinase (PKR) (S50216 [27]); MusPKR, mouse PKR (NP_035293 [28]); HumPKR, human PKR (A39650 [12]); DrosGCN2, *Drosophila* GCN2 (T13826 [29]); MusGCN2, mouse GCN2 (NP_038747 [30]); DrosPEK, *Drosophila* PEK (AAF61200 [31]); YeastGCN2, Saccharomyces cerevisiae GCN2 (P15442 [32]); CeleganPEK, *Caenorhabditis elegans* PEK (AAF61201 [31]); BeK, *B. mori* eIF-2α kinase; PfPK4, Plasmodium falciparum eIF-2α kinase-related enzyme (T28139 [33]). (B) Relationship between numbers of eIF-2α kinase family. The phylogenic tree was generated using PHYLIP program. The number above the branch points indicate bootstrap confidence level.

substrate, which suggests that eIF-2α phosphorylation by eIF-2α kinase is a central signaling event for the inhibition of general translation in response to various stress conditions [16]. However, the identity of the eIF-2α kinases involved in eIF-2α phosphorylation under specific stress conditions is largely unknown. More structural and functional information on various eIF-2α kinases is needed to better characterize the role of this kinase family.

To identify a novel eIF-2α kinase in *Bombyx mori*, an antisense degenerate oligonucleotide primer was synthesized based on the amino acids (KPSNV/IF) in conserved kinase domain VI: P1, 5′-AANAYNACRTTNSWNGGYTT-3′. We used λgt-10 forward or reverse primer as a sense primer. Polymerase chain reaction (PCR) was performed using 1 μM of P1 and 1 μM of λgt-10 forward or reverse primer as sense primer in 10 mM Tris–HCl, pH 8.0, 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM dNTP, and 2.5 units of *Thermus aquaticus* polymerase. An aliquot (5 μl) of λgt-10 *Bombyx* fat body library [17] was used as template DNA, after heating for 5 min at 70 °C. Seven specific PCR products of 200, 650, 750, 1100, 1150, 1200 and 1300 base pairs (bp) were generated. These fragments were subcloned and sequenced. The 1300-bp fragment displayed 30% homology to human PKR gene and was used to screen a cDNA library from *Bombyx* fat body. About 400,000 plaques were screened and six positive clones were isolated. One of these clones was subcloned into pBlue-Script SK(−) and sequenced. Sequencing analysis showed that this clone contained a 1959-bp insert composed of a 5′ noncoding region, an open reading frame of 1737 nucleotides, corresponding to 579 amino acids, and a 3′ untranslated region (Fig. 1). The deduced amino acid sequence has a NH_2 terminal regulatory domain and a kinase insert domain of 115 amino acids between kinase subdomains IV and V (Fig. 1). Sequence comparisons of the catalytic kinase domain with other known eIF2α kinases showed 29% identity with Yeast GCN2, 24% with *Drosophila* GCN2, 30% with all known HRIs (human, mouse, rat and rabbit), 26% with mammalian PKR and 23% with *Drosophila* PEK at the protein level (Fig. 2A). The NH_2 terminal regulatory domain of 138 amino acids did not show any significant similarity to known sequences by homology search of GenBank sequences. The kinase insertion domain of 115 amino acids was also unique, which along with the regulatory domain suggests a distinct function for this kinase. Sequence alignment analysis showed that the "Ile-Gln-Met-Xaa-Xaa-Cys" motif, known to be necessary for eIF2α phosphorylation, was conserved among all sequences (Fig. 2A). When we performed a phylogenetic tree analysis using Rat Erk2 protein as an outgroup, BeK protein was not classified among the previously known eIF-2α kinase family, suggesting that BeK is a novel eIF-2α kinase (Fig. 2B).

To investigate the tissue distribution of BeK, we performed RT-PCR analysis using different tissues from 5-day-old fifth instar larvae. The result showed that BeK was ubiquitously expressed in all tissues examined, including fat body, midgut, gonads, muscle, silk gland, epidermal tissue, Malpighian tubules and the dorsal aorta (Fig. 3). Interestingly, we found a significantly higher level of expression in the gonads.

Autophosphorylation is known to be an essential step for the activation of other eIF-2α kinase family [18]. To examine autokinase activity of endogenous BeK, BM-N cell line derived from *B. mori* larvae was used [19]. BEK from BM-N cell lysate was immunoprecipitated with BEK antiserum [antiserum directed against NH_2 terminal 12 amino acids peptide (KHSQDKWKALAT)] and the isolated immune complexes were incubated with γ-^{32}P-ATP in kinase assay buffer. The radiolabeled products were separated by SDS-PAGE and autoradiographed. The immunocomplex with BeK antiserum gave a single phosphorylated band, whereas no band was observed in an immunocomplex with preimmune serum (Fig. 4A). To examine the enzymatic activity of BeK, we first generated a BeK wild-type (BeKw) construct and a BeK catalytically inactive mutant (BeKm) construct that replaced Lys^{174} with Arg by PCR-based mutagenesis. Using these constructs, *Drosophila* Schneider cells stably expressing hexahistidine-tagged BeKw or hexahistidine-tagged BeKm under the control of metallothionine promoter were generated. In these cell lines, we can detect BeKw and BeKm with molecular mass of 65 kDa following copper induction, whereas no band was detected when uninduced cells were used (Fig. 4B). To investigate whether BeK can directly phosphorylate the alpha subunit of eIF2 as

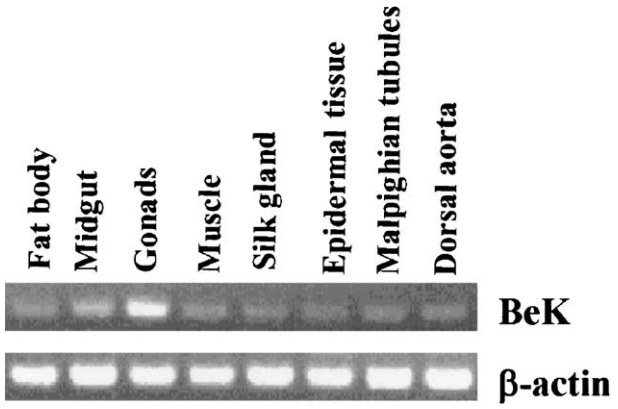

Fig. 3. RT-PCR analysis of BeK transcript in different fifth instar larval tissues. Total RNA from fifth instar larval tissues (fat body, midgut, gonads, muscle, silk gland, epidermal tissue, Malpighian tubules and dorsal aorta) was extracted and used for RT-PCR analysis. Isolated total RNA was treated with RNAse-free DNase, to avoid any chromosomal DNA contamination. First-strand cDNA was synthesized in 20 μl of reaction mixture containing 10 μg of total RNA, 1 mM dNTPs, 4 μg of random primers, 40 units of RNAsin, 10 units of AMV reverse transcriptase and 1 × buffer supplied by manufacturer. One microliter of cDNA was subjected to PCR amplification in 25 μl of reaction volume containing 200 μM dNTP, 4 mM $MgCl_2$, 1 μM of each primer and 0.5 Unit of *Taq* polymerase. The sequences of primers were as follows: BeK sense, 5′-CCG ACA GTG ACG AGT TTT CA-3′; BeK antisense, 5′-TCA TCG CTG TCA GAA ACC TG-3′; *B. mori* β-actin sense, 5′-CAC TGA GGC TCC CCT GAA C-3′ and antisense, 5′-GGA GTG CGT ATC CCT CGT AG-3′. For amplifying BeK mRNA, PCR of 26 cycles was used at 63 °C annealing temperature, whereas for *Bombyx* actin amplification, 22 cycles at 60 °C annealing temperature was employed.

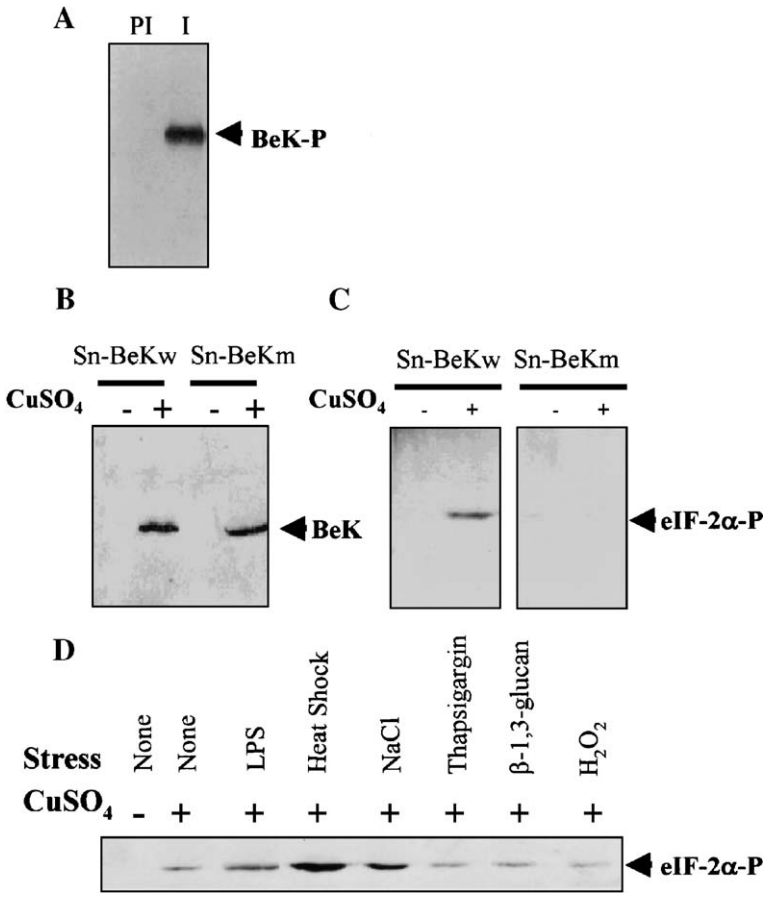

Fig. 4. Expression and kinase activity of BeK. (A) Autokinase and kinase activity of endogenous BEK from *Bombyx* BM-N cell line. Lysates from BM-N cells (2×10^6) were immunoprecipitated with preimmune serum (lane PI) and monospecific BeK antiserum (lane I) and autophosphorylation assay was performed in kinase assay buffer (25 mM HEPES, pH 7.4, 25 mM β-glycerophosphate, 25 mM $MgCl_2$, 2 mM dithiothreitol, 0.1 mM sodium orthovanadate) in the presence of γ-^{32}P-ATP (3000 Ci/mmol) at 30 °C for 30 min. Proteins were separated by SDS-PAGE and visualized by autoradiography. (B) Expression of BeK wild-type (BeKw) and BeK catalytically inactive mutant (BeKm) in insect cells. The hexahistidine-tagged full-length BeK open reading frame was subcloned into pMT/V5 vector (pMT/V5-His-BeKw) under control of metallothionin promoter (Invitrogen). The catalytically inactive mutant construct (pMT/V5-His-BeKm), which replaced Lys^{174} with Arg, was generated by PCR-based mutagenesis. Cell line stably expressing His-BeKw or His-BeKm was generated by transfection together with pCoHYGRO containing *Escherichia coli* hygromycin B-phosphotransferase gene under control of the *Drosophila copia* promoter (Invitrogen). Transfected cells were selected with hygromycin (300 μg/ml) for 6 weeks. Expression was induced in cells by addition of $CuSO_4$ to the culture media at a final concentration of 500 μM for 48 h, lysate from Schneider cells stably expressing BeKw (Sn-BeKw) or BeKm (Sn-BeKm) were subjected to Western blot analysis using specific anti-BeK antiserum at a dilution of 1:2000. After washing, the blot was incubated with goat anti-rabbit IgG conjugated to horseradish peroxidase diluted at 1:2000, and the signal was subsequently detected using ECL Western blot detection kit (Amersham). (C) eIF-2α kinase activity of BeK. Schneider cells stably expressing His-BeKw and His-BeKm were washed twice in Tris-buffered saline and solubilized in lysis buffer (20 mM HEPES, pH 7.4, 2 mM EDTA, 50 mM β-glycerophosphate, 1mM dithiothreitol, 1 mM sodium orthovanadate, 1% Triton X-100, 10% glycerol, 25 μg/ml aprotinin and 40 μg/ml phenylmethylsulfonyl fluoride). Equal amount of total protein was taken for immunoprecipitation reaction. The His-BeKw or His-BeKm was immunoprecipitated by incubating for 1 h at 4 °C with the monoclonal anti-His antibody (Qiagen) prebound to protein G-agarose for 15 min at room temperature. The immunoprecipitates were washed twice with RIPA buffer (50 mM Tris–Cl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate and 0.1% sodium dodecyl sulfate) and were finally washed once with kinase assay buffer (25 mM HEPES, pH 7.4, 25 mM β-glycerophosphate, 25 mM $MgCl_2$, 2 mM dithiothreitol, 0.1 mM sodium orthovanadate). The immunocomplexes were resuspended in 25 μl of kinase assay buffer containing 20 μM ATP and 1 μg of recombinant *Drosophila* eIF-2α subunit as substrate and incubated at 30 °C for 30 min. Reactions were terminated by adding Laemmli sample buffer and denatured in boiling water bath for 5 min. The reactions were then resolved by SDS-PAGE and analyzed by Western blot by using anti-phosphospecific eIF-2α antibody (Research Genetics) at a dilution of 1:2000. This antibody specifically recognizes the phosphorylated form of Ser^{51} of human eIF-2α and cross-reacts with the phosphorylated form of Ser^{50} of *Drosophila* eIF-2α. (D) Activation of BeK in response to various stress stimuli. *Drosophila* Schneider cells stably expressing His-BeKw were exposed to LPS (10 μg/ml for 30 min), heat shock (37 °C for 1 h), NaCl (300 mM for 30 min), thapsigargin (1 mM for 1 h), β-1,3-glucan (100 μg/ml for 30 min) and H_2O_2 (200 μM for 30 min). Following stress treatment, lysates were immunoprecipitated and immunocomplex kinase assay was performed using recombinant eIF-2α subunit as described in B. The phosphorylation of Ser^{50} of recombinant eIF-2α was visualized by Western blot analysis using anti-phosphospecific eIF-2α antibody as described in B.

substrate, a common substrate for eIF2α kinase family, cell lysate from Schneider cell expressing His-BeKw, was immunoprecipitated with monoclonal anti-His antibody, and a kinase assay was performed using the recombinant *Drosophila* eIF-2α subunit. The phosphorylation of the recombinant eIF-2α subunit was detected by Western blot analysis

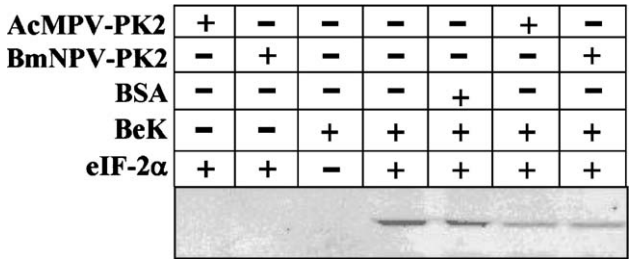

Fig. 5. Inhibition of eIF-2α phosphorylating activity of BeK by recombinant PK2. BeK was immunoprecipitated from Schneider cells expressing His-BeKw, and immunoprecipitates were subjected to kinase assay in the presence or absence of viral PK2. Recombinant PK2 (10 μg/reaction) from B. *mori* nuclear polyhedrosis virus (BmNPV-PK2) and from *A. californica* multiple nuclear polyhedrosis virus (AcMNPV-PK2) were used in this study. The same amount of bovine serum albumin (BSA) was used as control protein. Recombinant eIF-2α (1 μg) was used as substrate for BeK. Kinase assay and subsequent Western blot analysis using anti-phospho-specific eIF-2α antibody were performed as described in Fig. 4.

using antibody specifically recognizing the eIF-2α subunit phosphorylated at Ser^{50} (which corresponds to Ser^{51} in mammalian eIF2α). We found that BeKw can indeed phosphorylate the Ser^{50} site of eIF-2α subunit (Fig. 4C). In contrast to BeKw, BeKm showed no kinase activity (Fig. 4C) although it was expressed in equivalent amounts in cell lysate (Fig. 4B). These results strongly support our contention that BeK is involved in the general translational control system and that it acts by phosphorylating the Ser^{50} site of the eIF-2α subunit.

In *Drosophila*, it is well known that protein synthesis is dramatically inhibited in response to heat shock at 37 °C [20]. However, other stress stimuli leading to eIF-2α phosphorylation are largely unknown. To investigate whether BeK is involved in the eIF-2α phosphorylation in response to the above stress conditions, a BeK kinase assay was performed. *Drosophila* Schneider cells stably expressing BeKw were subjected to various eIF-2α phosphorylating forms of stress (immune stress, osmotic stress, heat stress, endoplasmic reticulum stress, and oxidative stress). BeKw was then immunoprecipitated and a kinase assay was performed, as described above. As shown earlier (Fig. 4C), the overexpression of BeKw in the absence of stress causes a basal level of eIF-2α phosphorylating activity. Of the different stress conditions, BeK is mainly activated in *Drosophila* Schneider cells following heat shock (37 °C for 1 h) and osmotic stress (300 mM NaCl for 30 min), and activated-BeK was shown to phosphorylate the eIF2α subunit at the Ser^{50} site (Fig. 4D). However, other stress conditions, such as immune stress (10 μg/ml LPS for 30 min or 100 μg/ml β-1,3-glucan for 30 min), endoplasmic reticulum stress (1 mM thapsigargin for 1 h) and oxidative stress (200 μM H_2O_2 for 30 min), could not significantly activate BeK activity. Densitometric analysis showed that heat stress and osmotic stress induced five-fold and three-fold increases in BeK enzymatic activity, respectively (data not shown).

The phosphorylation of eIF-2α by eIF-2α kinase results in the inhibition of general translational machinery, which is a common cellular mechanism for limiting protein synthesis under stress conditions. Many viruses have developed a number of strategies to down-regulate eIF-2α kinase to successfully propagate in host cells [21]. Insect viruses, such as *B. mori* nuclear polyhedrosis virus (BmNPV) and *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV), possess a truncated kinase referred to as PK2, which has been shown to inhibit eIF-2α phosphorylation in insect SF-9 cells [22]. However, it is unknown whether PK2 can inhibit the enzymatic activity of purified insect eIF-2α kinase. To investigate whether the insect viral gene product, PK2, inhibits the enzymatic activity of insect eIF-2α kinase, we performed an immunocomplex kinase assay in the presence of recombinant PK2. PK2 from BmNPV and from AcMPV, which share about 90% homology, were chosen for this investigation. We found that BeK enzymatic activity is significantly reduced in the presence of either BmNPV-PK2 or AcMNPV-PK2 versus BeK activity in the absence of viral PK2 (Fig. 5). No kinase activity was detected with viral PK2 alone. This result suggests that BeK may be one of the host targets of the viral eIF-2α kinase inhibitor, PK2. Further studies are necessary to elucidate the exact role of BeK in stress response and insect viral replication.

Acknowledgements

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