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Methods paper

A truncated *Danio rerio* PKZ isoform functionally interacts with eIF2α and inhibits protein synthesis

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ABSTRACT

A protein kinase containing Z-DNA binding domains (PKZ), which resembles protein kinase R (PKR) in domain organization, was recently discovered to be a member of the eIF2α kinase family in fish. PKR has roles in antiviral immunity through inhibiting protein synthesis and activating NF-κB; therefore, it is thought that PKZ may have a similar role in fish antiviral immunity. In the present study, the roles of two *Danio rerio* PKZ isoforms (*Dr*PKZ-A and *Dr*PKZ-B) in eIF2α phosphorylation and protein synthesis regulation were explored. *Dr*PKZ-A and *Dr*PKZ-B possess N-terminal Z-DNA binding domains and a conserved eIF2α kinase domain; however, they have domains of differing lengths inserted between kinase subdomains IV and V. *Dr*PKZ-A has an insert domain of 73 amino acids (aa), whereas *Dr*PKZ-B has an insert sequence of only 10 aa, suggesting that *Dr*PKZ-B could be a dysfunctional isoform or could interact with different substrates. Our results show that both *Dr*PKZ-A and *Dr*PKZ-B functionally interact with eIF2α and inhibit protein synthesis, although *Dr*PKZ-B possesses attenuated kinase activity. Our results also show that deletion of the insert in either isoform results in the complete abrogation of kinase activity, suggesting that the insert is critical for PKZ kinase activity. Kinase activity appears to be independent of insert length but may depend on the presence of specific amino acids within the insert domain. Furthermore, the effects of the N-terminal regulatory domain on kinase activity were analyzed. Deletion of the N-terminus results in reduced kinase activity of these isoforms relative to the wild-type forms, indicating that the isolated kinase domain is sufficient for eIF2α phosphorylation and that *Dr*PKZ-A and *Dr*PKZ-B may be regulated in a similar manner. Overall, our results show that *Dr*PKZ-B is a functional kinase in zebrafish and contribute to our understanding of the function of PKZ in fish.

1. Introduction

The eukaryotic initiation factor 2α (eIF2α) protein kinase family, which can phosphorylate eIF2α at Ser51, resulting in inhibition of protein synthesis initiation, plays an important role in regulating cell growth, proliferation, tumor development, and immune responses (Benne et al., 1978; Dever, 1999; Hinnebusch, 1994; Holcik and Sonenberg, 2005; Jagus et al., 1981; Ruggero and Pandolfi, 2003). eIF2 delivers initiator tRNAs to the translation machinery, and the phosphorylation of eIF2α reduces eIF2's activity by inhibiting the ability of eIF2 to exchange GDP for GTP, which is required for translation initiation (Kimball, 1999). The resultant reduction in global translation conserves energy and allows cells to reprogram gene expression to ameliorate damage caused by stress (Zaborske et al., 2010). A number of protein kinases belonging to the eIF2α kinase family have been identified and have been found to inhibit protein synthesis under certain conditions of environmental stress. For instance, a heme-regulated eIF2α kinase (HRI) exists in reticulocytes facing heme deficiency (Chen and London, 1995), a yeast GCN2 kinase coordinates the cellular response to uncharged tRNA (Zhu et al., 1996), RNA-regulated protein kinase (PKR)-like endoplasmic reticulum kinase (PERK) phosphorylates eIF2α under conditions of endoplasmic reticulum stress (Harding et al., 2000), and double-stranded RNA-regulated protein kinase (PKR) is activated by dsRNA (Clemens and Elia, 1997; Clemens et al., 1993; Langland et al., 1996).

The members of the eIF2α kinase family are characterized by a common domain organization, which consists of a regulatory domain that senses distinct stress stimuli and a conserved serine/threonine (Ser/Thr) protein kinase domain, which can be activated by autophosphorylation upon binding of the regulatory domain to the

Abbreviations: PKZ, a protein kinase containing Z-DNA binding domains; PKR, protein kinase R; eIF2α, eukaryotic initiation factor 2α; HRI, heme-regulated eIF2α kinase; PERK, RNA-regulated protein kinase (PKR)-like endoplasmic reticulum kinase; GCN2, general control nonrepressed-2; ZBD, Z-DNA binding domain; ADAR1, double-stranded RNA adenosine deaminase; co-IP, co-immunoprecipitation; ISKNV, infectious spleen and kidney necrosis virus; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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appropriate substrate (Zhan et al., 2004). Based on multiple amino acid sequence alignments, the kinase domains can be further divided into 12 smaller subdomains defined by regions that contain characteristic patterns of conserved residues along with an insert domain, which are uninterrupted by large amino acid insertions (Hanks and Hunter, 1995). Although there is sequence similarity among the catalytic domain of the eIF2α kinases and other serine/threonine kinases, a unique feature that distinguishes eIF2α kinases from other kinases is the presence of a large spacer known as the kinase insert domain, which lies between kinase subdomains IV and V (Hanks and Hunter, 1995).

A protein kinase containing Z-DNA binding domains (PKZ), which contains a conserved eIF2α kinase domain in its C-terminus and two Z-DNA binding domains within its N-terminal regulatory domain, is another recently discovered member of the eIF2α kinase family in fish (Hu et al., 2004; Rothenburg et al., 2005). In 2004, a PKZ isoform isolated from *Carassius auratus* (CaPKR-like gene) was first identified in IFN-producing CAB cells after treatment with UV-inactivated GCHV (Hu et al., 2004). In subsequent studies, cDNAs encoding PKZ homologs were cloned from Zebrafish (*Danio rerio*), Atlantic salmon (*Salmo salar*), and rare minnow (*Gobiocypris rarus*), among other species (Bergan et al., 2008; Rothenburg et al., 2005; Su et al., 2008). The Z-DNA binding domain (ZBD) found in PKZs is also found in double-stranded RNA adenosine deaminase (ADAR1), Z-DNA binding protein 1, and vaccinia virus E3L (Herbert et al., 1997; Kim et al., 2004; Schwartz et al., 2001). ZBDs bind tightly and specifically to Z-DNA, and the ZBDs of PKZ from *C. auratus* have been shown to interact with d(GC)n and form a complex with Z-DNA *in vitro* (Ha et al., 2008; Kim et al., 2009; Wu et al., 2010). The C-terminal kinase domain of PKZ is highly homologous to the eIF2α kinase, and has been shown to phosphorylate eIF2α *in vitro* and to inhibit protein synthesis *in vivo* (Bergan et al., 2008; Rothenburg et al., 2005).

D. rerio PKZ (*Dr*PKZ), variants A through D (named in order of decreasing abundance), were reported by Rothenburg and colleagues in 2005 (Rothenburg et al., 2005). Variant A (*Dr*PKZ-A), which encodes the entire protein and contains an insert domain of 78 aa, binds to Z-DNA *in vitro* and is thought to inhibit protein synthesis, based on the results of a co-transfection assay (Rothenburg et al., 2005). By contrast, the functions of *Dr*PKZ-B, which lacks most of the insert sequence, remain unknown. This study aims to elucidate the specific roles of *Dr*PKZ-B *in vivo*.

In the present study, we amplified *Dr*PKZ-A and *Dr*PKZ-B using a template from infectious spleen and kidney necrosis virus (ISKNV)-infected zebrafish spleen samples and then investigated whether the isoforms functionally interact with eIF2α and inhibit protein synthesis *in vivo*. Our findings indicate that both *Dr*PKZ-A and *Dr*PKZ-B functionally interact with eIF2α and inhibit protein synthesis *in vivo*, and deletion of the insert domain from either *Dr*PKZ-A or *Dr*PKZ-B results in the complete abrogation of kinase activity. Deletion of the N-terminus leads to attenuated kinase activity relative to the wild-type isoform.

2. Materials and methods

2.1. Antibodies and reagents

Mouse monoclonal anti-V5 antibody was purchased from Invitrogen (USA). Rabbit polyclonal anti-Myc antibody, polyclonal rabbit anti-actin, anti-mouse IgG secondary antibody, and anti-rabbit secondary antibody conjugated to alkaline phosphatase were purchased from Sigma (USA). Rabbit anti-eIF2α antibody and rabbit anti-phospho-eIF2α (Ser51) antibody were purchased from Cell Signaling Technology (USA). Chromogenic detection reagents for alkaline phosphatase were obtained from Roche (Switzerland) and WeiJia (China). Dual-Glo® Luciferase Reporter Assay System was purchased from Promega (USA).

2.2. Zebrafish and viral infection

Wild-type zebrafish were cultivated in a recirculating system at 28 °C with a natural photoperiod. Zebrafish were fed twice daily with commercial pellets as previously described (Xu et al., 2008). Male and female adult fish (3 months old) used in the infection experiments were transferred to an isolated flow-through system and allowed to acclimate to the system for 7 days prior to ISKNV infection. All fish were similarly anesthetized prior to ISKNV injection and sample collection.

2.3. Plasmid constructs

*Dr*PKZ-A and *Dr*PKZ-B were amplified by PCR from a cDNA template obtained from zebrafish spleen tissue. The oligonucleotide primers used for the recombinant vectors are listed in Table 1, and maps of the cloned segments are illustrated in Fig. 1. Deletion mutations were generated based on our previous knowledge of similarities between PKR and PKZ (Rothenburg et al., 2005). The cDNA sequences encoding the full-length and truncated *Dr*PKZ isoforms were amplified using forward primers containing a BamHI site and reverse primers containing an EcoRI site. The reverse primers were complementary to the 3′ end of *Dr*PKZ and were engineered to omit the stop codon, allowing the ribosome to read through. The PKZ-F/-R primer pair was used to amplify the entire lengths of *Dr*PKZ-A and *Dr*PKZ-B, whereas the PKZ-F475/PKZ-R primer pair was used to amplify the putative kinase domains of *Dr*PKZ-A and *Dr*PKZ-B and to delete the N-terminal Z-DNA binding domains. The middle truncated segment ΔID of *Dr*PKZs, a mutant whose putative insert domain is deleted, was obtained by overlap PCR (Higuchi et al., 1988). Briefly, two overlapping DNA fragments corresponding to the sequence encoding the upstream section (the sequence before the insert domain) and the downstream segment (the sequence after the insert domain) of PKZ were obtained using the primer pairs PKZ-F/ΔID-R and ΔID-F/PKZ-R, respectively. The products were gel purified and used as templates to amplify the truncated segments using the PKZ-F/-R primer pair. The amplified products were cleaved with BamHI and EcoRI and were cloned in frame into the BamHI/EcoRI sites within the pcDNA3.1 vector (Invitrogen), to produce a series of full-length and truncated PKZs-3.1a plasmids expressing C-terminal V5-tagged proteins. cDNA from zebrafish was used as the template for PCR amplification with the primer pair eIF2-F/eIF2-R; the product was cut using EcoRI and KpnI and then cloned into the pCMV-Myc vector to obtain a recombinant vector expressing eIF2α for zebrafish with a c-Myc tag. All plasmids were confirmed by sequencing analysis.

2.4. Real-time quantitative PCR

To measure the tissue distribution of the *Dr*PKZ-A and *Dr*PKZ-B *in vivo*, the expression profiles of *Dr*PKZ-A and *Dr*PKZ-B mRNA were analyzed by real-time quantitative RT-PCR using SYBR Green I and the LightCycler 480 System (Roche, Switzerland). RNA samples extracted from 10 dissected tissues from 15 fish were reverse-transcribed (RT) into cDNA (Xiang et al., 2010). The expression patterns were analyzed by real-time PCR using specific primers listed in Table 1. Expression levels were normalized to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene transcription level. The PCR reaction contained a final volume of 10 μL, which consisted of 1 μL of the cDNA reaction (1:10 dilution), 5 μL of SYBR Green I mix (Toyobo, Japan), 0.2 μL of each primer (sense and antisense, 10 μM), and 3.6 μL of ddH_2O. The reaction was heated to 95 °C for 2 min and then run through the PCR program (15 s at 94 °C, 15 s at 60 °C and 30 s at 72 °C) for 40 cycles. Fluorescence data for quantification was collected at 75 °C. Melting curve analysis was performed over a range of 60–95 °C to verify single product amplification upon completion of the assay.