Identification of the dual specificity and the functional domains of the cardiac-specific protein kinase TNNI3K

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Abstract. Molecular cloning of cardiac troponin I-interacting kinase (TNNI3K), a novel cardiac-specific protein kinase containing seven N-terminal ankyrin (ANK) repeats followed by a protein kinase domain and a C-terminal Ser-rich domain, has previously been reported. In the present study, we show that the C-terminal functional region of TNNI3K negatively regulates the kinase activity, and the N-terminal ANK domain is necessary for autophosphorylation. An *in vitro* kinase assay shows that TNNI3K exhibits dual-specific kinase activity and forms dimers or oligomers that may be necessary for its activation.

Key words: Protein kinase — Mixed lineage kinase — Cardiac troponin I-interacting kinase — Ankyrin repeats — Ser-rich domain

Introduction

Protein kinases mediate most signal transduction processes in eukaryotic cells; by modifying the activities of their substrates, they also control many other cellular processes including metabolism, transcription, cell cycle progression, cytoskeletal rearrangement, cell movement, apoptosis and differentiation. Protein phosphorylation also plays a critical role in intercellular communication during development, in physiological responses, in homeostasis, and in the functioning of the nervous and immune systems (Manning et al. 2002).

We previously described the cloning of a novel gene that encodes a cardiac-specific protein kinase, designated cardiac troponin I-interacting kinase (TNNI3K) (GenBank accession No. AF116826) (Zhao et al. 2003). TNNI3K contains three kinds of domains: seven N-terminal ankyrin (ANK) repeats, a protein kinase domain containing primary sequence motifs conserved in both serine/threonine and tyrosine protein kinases, and a C-terminal serine-rich (Ser-rich) domain. Sequence comparison of catalytic domains, together with sequence similarities and domain structures outside the catalytic domain, assigns TNNI3K to a new family of protein kinases, the mixed lineage kinase (MLK) family in the tyrosine kinase-like group (Manning et al. 2002). The MLKs comprise a recently-described protein kinase family consisting of MLK1-4, dual leucine zipper-bearing kinase (DLK), leucine zipper-bearing kinase (LZK), leucine zipper and sterile-α motif kinase, TGF-β-activated kinase-1 and integrin-linked kinase (ILK) (Sakuma et al. 1997; Liu et al. 2000; Manning et al. 2002). Kinases in this family share a structural feature, the kinase catalytic domain, which contains the characteristic amino acid sequences of both serine/threonine and tyrosine kinases. Several MLK family proteins have been proposed to function as mitogen-activated protein kinase kinase kinases (MAP3Ks) (Yamaguchi et al. 1995; Fan et al. 1996; Rana et al. 1996; Hirai et al. 1997; Sakuma et al. 1997; Liu et al. 2000; Bloem et al. 2001). However, little is known about the overall biochemical and functional roles of MLKs.

Most protein kinases act in a network of kinases and other signaling effectors and are modulated by autophosphorylation and by phosphorylation by other kinases (Manning et al. 2002). Other domains within these proteins may regulate the kinase activity, link to other signaling modules, or local-

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ize the protein subcellularly (Manning et al. 2002). TNNI3K has two domains in addition to the kinase domain, and the significance of these merits investigation.

Many MAP3Ks such as apoptosis signal-regulating kinase 1 (ASK1) (Gotoh and Cooper 1998), including MLKs (Mata et al. 1996; Leung and Lassam 1998; Liu et al. 2000; Nihalani et al. 2000; Vacratsis and Gallo 2000; Ikeda et al. 2001), form dimers or oligomers. Dimerization/oligomerization is believed to be necessary for activation of the downstream signal transduction. Thus, in the present study, we have examined whether TNNI3K forms dimers or oligomers.

It was previously reported that TNNI3K can undergo autophosphorylation (Zhao et al. 2003). In the present study, we confirmed the protein kinase activity by an *in vitro* assay using myelin basic protein (MBP) as substrate, and we identified the functional domains using serial deletion mutants of TNNI3K. Furthermore, it was demonstrated that TNNI3K has dual-specificity kinase activity. Finally, it was discovered that TNNI3K forms dimers or oligomers when over-expressed.

Materials and Methods

Plasmids, antibodies and reagents

To construct a mammalian expression vector for FLAG-tagged TNNI3K (FLAG-TNNI3K), the cDNA fragment encoding the open reading frame of TNNI3K was subcloned in-frame into the pcDNA6-FLAG vector. Expression plasmids for deletion mutants of TNNI3K were constructed with the pcDNA6-FLAG vector, and cDNAs encoding different parts of TNNI3K are described below. To construct pcDNA6-FLAG/TNNI3K-PK, a polymerase chain reaction (PCR)-amplified fragment encoding the same part of the TNNI3K molecule was digested with *Kpn* I and *BamH* I and was subcloned into the pcDNA6 vector using the following primers: 5'-AGGGTACCGAGATTATTGGCTCAGGTTC-3' (sense primer) and 5'-GAGGATCCCTCAATGTTGCAGAGACACTC-3' (antisense primer). Likewise, to construct an expression plasmid for a mutant TNNI3K lacking the C-terminal Ser-rich domain, pcDNA6-FLAG/TNNI3KΔSer, the cDNA fragment of TNNI3K encoding amino acids 1 to 727 was amplified by PCR and then inserted into the *Kpn* I-*BamH* I fragment of pcDNA6-FLAG: 5'-TAGGTACCATGGGAAATTATAAATCTAGAC-3' (sense primer) and 5'-GAGGATCCCTCAATGTTGCAGAGACACTC-3' (antisense primer). An expression plasmid for a mutant TNNI3K that lacks the N-terminal ANK-motif, pcDNA6-FLAG/TNNI3KΔANK, was constructed similarly using the following primers: 5'-AGGGTACCGAGATTATTGGCTCAGGTTC-3' (sense primer) and 5'-GTGGATCCCGAATGCTGTCAGCTGCTG-3' (antisense primer), which were used to amplify the cDNA fragment of TNNI3K encoding amino acids 463 to 835. The expression constructs pGEX-5X-1/TNNI3K and pcDNA4-Xpress/TNNI3K were described previously (Zhao et al. 2003). Anti-FLAG and anti-Xpress antibodies were purchased from Sigma and Invitrogen, respectively; anti-p-Tyr (PY99) was from Santa Cruz.

Cell culture and transfection

Human embryonic kidney cell line HEK-293FT cells were cultured in DMEM supplemented with 10% fetal calf serum. For transfection, cells were subcultured and grown overnight and then transiently transfected with various expression constructs using Lipfectamine 2000 (Invitrogen) according to the manufacturer's protocol. The cells were lysed in SDS-PAGE sample buffer at 48 h post-transfection and the lysates were subjected directly to SDS-PAGE followed by Western blotting. Alternatively, the cells were lysed at 48 h post-transfection and the lysates were subjected to immunoprecipitation and Western blotting.

Western blotting

Total proteins were separated under reducing conditions on SDS-PAGE and then electrically transferred on to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked for 1 h in Tris-buffered saline (TBS, pH 7.5) containing 5% nonfat dry milk, followed by incubation with the anti-FLAG antibody or PY99 antibody diluted in blocking buffer, then probed with appropriate horseradish peroxidase-conjugated second antibodies. Blots were developed using an enhanced chemiluminescence detection system (Amersham Pharmacia).

In vitro kinase activity

Cells were transfected with expression plasmids. After 48 h, lysates were prepared in lysis buffer comprising 1% NP-40, 0.25% sodium deoxycholate (DOC), 1 mmol/l EGTA, 1 mmol/l EDTA, 150 mmol/l NaCl, 50 mmol/l Tris-HCl, pH 7.5, 1 mmol/l Na_3VO_4, 100 mmol/l NaF, 1 mmol/l β-glycerophosphate, 5 mg/ml Protease Inhibitor Cocktail Tablets (complete mini, Roche). After centrifugation, the clear lysates were immunoprecipitated with anti-FLAG antibody and Protein G-Agarose (Roche). The beads were washed three times with the lysis buffer and twice with kinase reaction buffer (25 mmol/l Tris-HCl, pH 7.5; 10 mmol/l $MgCl_2$; 10 mmol/l $MnCl_2$; 5 mmol/l β-glycerophosphate; 2 mmol/l dithiothreitol. The immunoprecipitates were incubated with a kinase reaction buffer containing 10 μCi of [γ-^{32}P] ATP and 2 μg MBP for 30 min at 30°C. Reactions were terminated by adding SDS-PAGE sample buffer, then the proteins were resolved by SDS-PAGE and phosphorylated substrates were visualized by autoradiography.

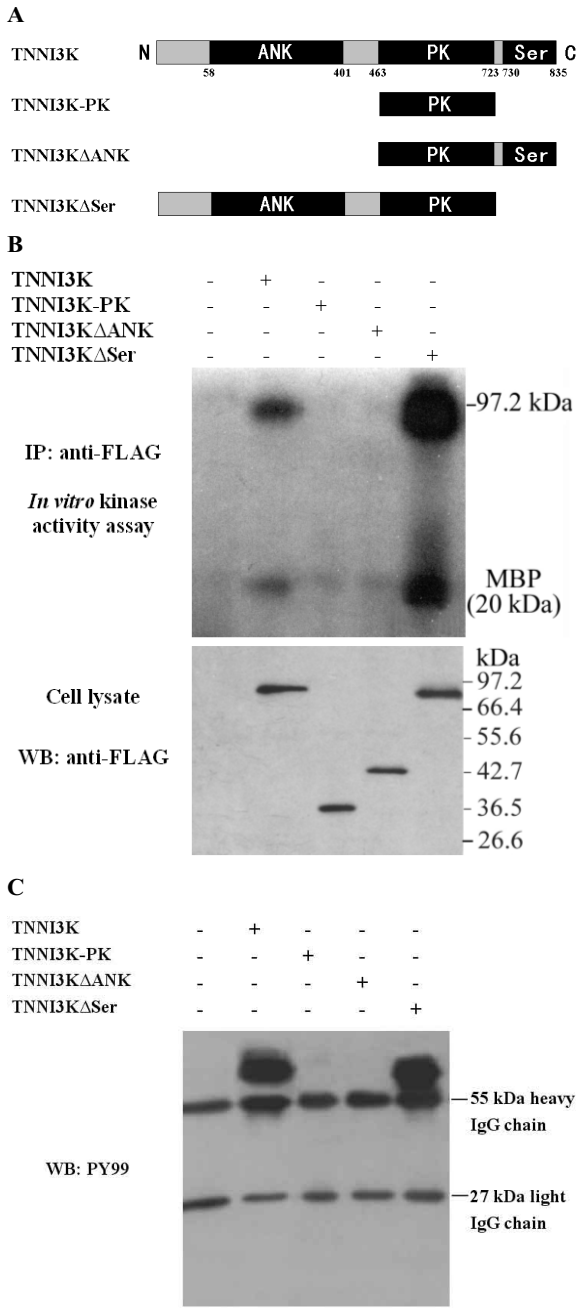

Figure 1. Cardiac troponin I-interacting kinase (TNNI3K) phosphorylates myelin basic protein (MBP) and also autophosphorylates. **A.** FLAG-tagged TNNI3K (FLAG-TNNI3K) and its serial mutants used in this study are schematically represented. **B.** The kinase activities of TNNI3K and serial TNNI3K mutants were measured by an *in vitro* assay with MBP as a substrate, while the expressions of TNNI3K and serial FLAG-TNNI3K mutants were confirmed by Western blotting (WB) with anti-FLAG antibody. **C.** After autoradiography, the proteins were electrically transferred on to polyvinylidene difluoride (PVDF) membranes and the phosphorylated tyrosines were immunodetected with anti-p-Tyr (PY99) antibody. ANK, ankyrin repeats domain; PK, protein kinase domain; Ser, serine-rich domain; IgG, immunoglobulin G; N, N-terminal; C, C-terminal; IP, immunoprecipitation.

Immunoprecipitation and Western blotting

Cells transiently transfected with various plasmids were lysed in cell lysis buffer (1% NP-40, 0.25% DOC, 2 mmol/l EGTA, 1 mmol/l EDTA, 150 mmol/l NaCl, 50 mmol/l Tris-HCl, pH 7.5, 5 mg/ml Protease Inhibitor Cocktail Tablets). After centrifugation, the clear lysate was subjected to immunoprecipitation. Briefly, the lysates were incubated with 2 μg anti-FLAG antibody for 6 h at 4°C, then mixed with 20 μl protein G-agarose followed by incubation for an additional 3 h. Immunoprecipitates were collected by centrifugation and washed with washing buffer as instructed by the manufacturer's protocol. Total proteins were then separated by SDS-PAGE and specific protein bands were visualized using the anti-Xpress antibody and developed as described above.

In vitro binding assay

The glumthione S transferase (GST)-fused TNNI3K (GST-TNNI3K) was produced in *E. coli* using the pGEX expression system (Amersham Pharmacia). The GST-TNNI3K protein was affinity-purified on glutathione-sepharose beads following the manufacturer's protocol but without the final elution; GST protein served as the control. The beads were then added to an equal volume of lysate of HEK-293FT cells transfected with a plasmid that expresses Xpress-tagged TNNI3K (Xpress-TNNI3K). The reaction mixture was incubated on ice for 3 h. The beads were subsequently washed in phosphate buffered saline and isolated proteins were subjected to SDS-PAGE followed by immunoblot analysis using anti-Xpress antibody.

Results

TNNI3K is an active protein kinase

Previous observations demonstrated that TNNI3K autophosphorylates, indicating that it is a functional protein kinase (Zhao et al. 2003). To confirm this further, anti-FLAG antibody immunoprecipitates of TNNI3K transfectants were incubated with the substrate MBP in the presence of [γ-^{32}P]ATP, Mn^{2+} and Mg^{2+}. The proteins were resolved by SDS-PAGE under reducing conditions and radiolabeled proteins were visualized by autoradiography. In addition to the autophosphorylated band at 93 kDa, a band at 20 kDa was detected in the TNNI3K transfectants; there were no labeled bands in non-transfectants (Fig. 1B). The 20 kDa band comigrated with MBP, indicating that TNNI3K phosphorylates not only itself but also MBP.

To clarify whether the other two domains regulate the kinase activity, *in vitro* kinase assays were performed using immunoprecipitates of the series of FLAG-TNNI3K

mutants. Using immunoprecipitates of TNNI3KΔSer transfectants, a mutant TNNI3K that lacks the C-terminal Ser-rich domain, two radioactive bands were visualized: one at 20 kDa, indicating the MBP substrate, and the other at around 90 kDa, which is consistent with the molecular weight of the recombinant protein. That is, TNNI3KΔSer can also phosphorylate both MBP and itself just as TNNI3K does. As shown in Fig. 1B, both the amount of autophosphorylation and the amount of MPB phosphorylation in TNNI3KΔSer was much greater than in TNNI3K. This suggests that the C-terminal Ser-rich domain has a strong inhibiting effect on the kinase activity of TNNI3K when over-expressed.

While the immunoprecipitates of TNNI3K and TNNI3KΔSer could autophosphorylate, the other two deletion mutants, TNNI3K-PK and TNNI3KΔANK, which lack the N-terminal ANK domain, showed no autophosphorylated bands. These data indicate that the N-terminal ANK domain is necessary for TNNI3K autophosphorylation.

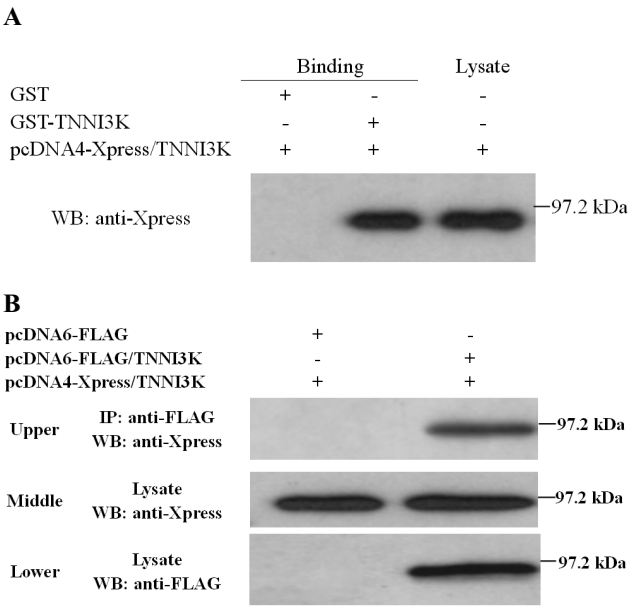

Figure 2. Cardiac troponin I-interacting kinase (TNNI3K) forms dimers or oligomers. **A.** *In vitro* binding assay. Equal amounts of the lysate from HEK-293FT cells transfected with pcDNA4-Xpress/TNNI3K were added to glumthione S transferase (GST)- and GST-fused TNNI3K (GST-TNNI3K)-glutathione-sepharose beads. Proteins bound to the beads were finally analyzed by immunoblot analysis with anti-Xpress antibody. **B.** Xpress-tagged TNNI3K (Xpress-TNNI3K) was expressed with or without FLAG-tagged TNNI3K (FLAG-TNNI3K) in HEK-293FT cells and FLAG-TNNI3K was immunoprecipitated (IP) from each cell lysate. The presence of Xpress-TNNI3K in the immunoprecipitate was examined by Western blotting (WB) with anti-Xpress antibodies (upper). The expressions of Xpress-TNNI3K and FLAG-TNNI3K in cells were examined by immunoblotting with anti-Xpress or anti-FLAG antibodies, respectively (middle and lower).

TNNI3K is a dual-specificity protein kinase

To determine whether TNNI3K is a tyrosine kinase, the proteins from the *in vitro* kinase activity assay were electrically transferred on to PVDF membranes and subjected to Western blotting using PY99 as the primary antibody. Strong immunoreactive bands were detected at the sites of autophosphorylation of TNNI3K and TNNI3KΔSer by comparison with the autoradiograms, suggesting that TNNI3K has tyrosine kinase activity (Fig. 1C). In contrast, no band was found at the site MBP phosphorylation. Taken together, these results indicate that TNNI3K is a dual-specificity protein kinase.

TNNI3K forms dimers or oligomers

Since almost all the MLKs form dimers or oligomers, it is likely that TNNI3K can also form homo-dimers or oligomers. This interaction was investigated using an *in vitro* binding assay. GST or GST-TNNI3K bound to glutathione-sepharose beads was added to a lysate of HEK-293FT cells transfected with pcDNA4-Xpress/TNNI3K (Fig. 2A). After extensive washing, proteins bound to the beads were precipitated and analyzed by immunoblotting with anti-Xpress antibody. The results indicated that Xpress-TNNI3K in the lysate was retained by the GST-TNNI3K-glutathione-sepharose beads but not by the GST-glutathione-sepharose beads, suggesting that TNNI3K interacts with Xpress-TNNI3K.

To determine further whether TNNI3K forms dimers or oligomers in cells, a co-immunoprecipitation assay was carried out. FLAG-TNNI3K was co-expressed with Xpress-TNNI3K in HEK-293FT cells, followed by immunoprecipitation with anti-FLAG antibodies, and the co-immunoprecipitated Xpress-TNNI3K was detected by Western blotting with anti-Xpress antibodies as the primary antibodies. As shown in Fig. 2B, Xpress-TNNI3K co-immunoprecipitated with FLAG-TNNI3K, indicating that TNNI3K forms homo-dimers or oligomers in cells.

Discussion

Negative regulation by the C-terminal Ser-rich domain

The Ser-rich domain is a common feature of protein kinases; its function is currently obscure. However, it has been shown that this domain may participate in the regulation of kinase function. In the death-associated protein kinase, the C-terminal Ser-rich tail normally plays a negative regulatory role

(Raveh et al. 2000; Shohat et al. 2001). The Ser-rich region of 3-phosphoinositide-dependent kinase 1 is essential for its sphingosine-stimulated autophosphorylation, which in turn modifies the enzymatic activity (King et al. 2000). Moreover, kinase from chicken, a Ste20-like kinase with mitogenic potential and ability to activate the stress-activated protein kinase/c-Jun NH_2-terminal kinase pathway, failed increase cell growth rate significantly after its Ser-rich region was deleted (Yustein et al. 2000). The N-terminal serine cluster of Ca^{2+}/calmodulin-dependent protein kinase type IV (CaMK IV) mediates a novel type of intrasteric inhibition (Chatila et al. 1996).

A similar regulatory function was revealed by our data that the C-terminal Ser-rich domain has a strong inhibiting effect on TNNI3K kinase activity when over-expressed. The biological activity of this tail could hypothetically be modeled so that phosphorylation of the serine motif by other kinases may coordinates the control of kinase activation. However, we failed to find any TNNI3K-interacting kinases when we used a yeast two-hybrid screen with the Ser-rich domain as bait (Zhao et al. 2003).

Dimerization or oligomerization may be necessary for autophosphorylation and activation

TNNI3K and other MLK family members are constitutively active when over-expressed (Bloem et al. 2001). TNNI3K simultaneously forms dimers or oligomers and performs autophosphorylation when over-expressed in cells. Thus, TNNI3K may undergo autophosphorylation to regulate its activity upon upstream stimulation. In this event, autophosphorylation is similar to receptor tyrosine kinase activation. Upon ligand binding, these receptors form homo-dimers and phosphorylate their dimerized partners at tyrosine residues, resulting in kinase activation. Similar autophosphorylation mechanisms have been investigated for MAP3Ks such as ASK1 (Gotoh and Cooper 1998) and MLK3 (Leung and Lassam 1998; Vacratsis and Gallo 2000). Our data show that the amount of MBP phosphorylated by proteins capable of autophosphorylation was much more than by proteins incapable of autophosphorylation, especially when the C-terminal inhibition was lacking. It therefore appears that the amount of autophosphorylation of TNNI3K parallels its kinase activity, suggesting that autophosphorylation participates in activation. Our results in this report clearly demonstrate that the N-terminal ANK domain is necessary for TNNI3K autophosphorylation. Since the ANK domain is commonly implicated in protein-protein interactions, it is reasonable to suspect that this domain is responsible for the dimerization or oligomerization of TNNI3K, which can be directly confirmed by the co-immunoprecipitation assay using the series of TNNI3K deletion mutants.

The dual specificity of TNNI3K

Most MLKs are serine/threonine protein kinases, such as MLK7 (Bloem et al. 2001), LZK (Sakuma et al. 1997), DLK (Holzman et al. 1994; Mata et al. 1996), ILK (Hannigan et al. 1996) and MLK3 (Gallo et al. 1994). However, here we have demonstrated that TNNI3K, a new member of the MLK family, has a dual-specificity kinase activity. It may be autophosphorylated at tyrosine residues in a manner similar to activated receptor tyrosine kinases, but at the same time it phosphorylates substrates at serine/threonine residues. However, the present experiments cannot exclude the possibility that some serine/threonine residues are phosphorylated during autophosphorylation and that TNNI3K might phosphorylate some substrates at tyrosine residues.

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