ISSN 0006-2979, Biochemistry (Moscow), 2007, Vol. 72, No. 11, pp. 1199-1204. © Pleiades Publishing, Ltd., 2007.
Published in Russian in Biokhimiya, 2007, Vol. 72, No. 11, pp. 1474-1481.
Originally published in Biochemistry (Moscow) On-Line Papers in Press, as Manuscript BM07-087, July 29, 2007.

AOP-1 Interacts with Cardiac-Specific Protein Kinase TNNI3K and Down-regulates Its Kinase Activity

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Received April 2, 2007
Revision received June 4, 2007

Abstract—In the present study, a yeast two-hybrid screening system was used to identify the interaction partners of cardiac troponin I-interacting kinase (TNNI3K) that might serve as regulators or targets, and thus in turn to gain some insights on the roles of TNNI3K. After screening the adult heart cDNA library with a bait construct encoding the ANK motif of TNNI3K, antioxidant protein 1 (AOP-1) was isolated. The interaction between TNNI3K and AOP-1 was confirmed by the *in vitro* binding assay and coexpression experiments *in vivo*. The colocalization of TNNI3K and AOP-1 was clarified by confocal immunofluorescence. Moreover, coexpression of AOP-1 inhibited TNNI3K kinase activity in the *in vitro* kinase assay.

DOI: 10.1134/S0006297907110053

Key words: AOP-1, protein kinase, protein–protein interaction, TNNI3K, yeast two-hybrid system

Protein phosphorylation is a regulatory tool most commonly used in eukaryotic cells. The number of kinases that are recognized as members of signal transduction pathways or as key regulators of specific processes such as cell cycle progression, transcription, replication, mitosis, metabolic processes, etc. is steadily increasing. The completion of the human genome sequence now allows the identification of almost all human protein kinases, and 518 putative protein kinase genes were identified [1]. With every newly discovered enzyme, the task is to identify its physiological substrates, upstream regulators, downstream targets, and finally its biological role.

We have recently cloned a novel kinase gene, TNNI3K (cardiac troponin I-interacting kinase), from an adult cDNA library. Northern blot analysis demonstrated that it is cardiac-specific [2]. TNNI3K protein contains three kinds of domains: seven N-terminal ankyrin repeats (ANK) followed by a protein kinase domain, with primary sequence containing motifs conserved in both serine/threonine and tyrosine protein kinases, and a C-terminal Ser-rich domain. It belongs to a new kinase family, the mixed lineage kinase (MLK) family in the tyrosine kinase-like (TKL) group, according to the sequence comparison of the catalytic domain aided by knowledge of sequence similarity and domain structure outside of the catalytic domain [1]. It was found that TNNI3K can undergo autophosphorylation [2]. However, the precise biological functions of TNNI3K are not yet quite understood.

In the present investigation, the interaction partners of TNNI3K that might function as upstream regulators or as downstream targets were sought. Since the N-terminal ANK-containing domain, among the three domains of TNNI3K, is one of the most common domains mediating additional protein–protein interactions, a yeast two-hybrid system was performed to identify proteins that interact with TNNI3K using a bait construct encoding the ANK motif of it. Several clones were isolated and one

Abbreviations: ANK) ankyrin repeats; AOP-1) antioxidant protein 1; GFP) green fluorescent protein; MBP) myelin basic protein; ROS) reactive oxygen species; TNNI3K) cardiac troponin I-interacting kinase.

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of these clones encoded a mitochondrial antioxidant protein, antioxidant protein 1 (AOP-1) [3]. The interaction between AOP-1 and TNNI3K was investigated in greater detail. Strikingly, coexpression of AOP-1 and TNNI3K lead to the decreasing of the kinase activity of TNNI3K.

MATERIALS AND METHODS

Plasmids, antibodies, and reagents. The expression constructs of pcDNA6-FLAG/TNNI3K, a series of FLAG-tagged TNNI3K deletion mutants, were described elsewhere [4]. As the plasmid pACT2/AOP-1, which was selected from the human heart cDNA library, missed the coding sequence for the first four amino acids on the N-terminal of human AOP-1, the full-length cDNA for the AOP-1 coding region was amplified by PCR with a sense primer containing the missing 12 bp oligonucleotides and then subcloned in-frame into the pcDNA4-Xpress vector (Invitrogen Co., USA) to construct a mammalian expression vector for AOP-1. The sequences of the primers used were: 5′-CAGATATC-CATGGCGGCTGCTGTAGGACGGTTGCTCCG-3′ (sense primer) and 5′-CCGCTCGAGCTGATTTAC-CTTCTGAAAGTACTC-3′ (antisense primer). To construct an expression plasmid for a deletion mutant of pcDNA4-Xpress/AOP-1ΔN, lack of the N-terminal amino acids 1-60, the cDNA fragment was amplified by PCR and then inserted into the *EcoR*I/*Xho*I sites of pcDNA4-Xpress, using the following primers: 5′-CTGAATTCTGGCACCTGCTGTCACCCAGCATG-CAC-3′ (sense) and 5′-CCGCTCGAGCTGATTTAC-CTTCTGAAAGTACTC-3′ (antisense). The expression vectors for antioxidation-negative AOP-1 point mutants were constructed by PCR-based site-directed mutagenesis, TGT (C108) being converted into AGT (Ser) in the C108S mutant, and TGC (C229) into AGC (Ser) in the C229S mutant. The sequences of the oligonucleotides used for C108S and C229S were: 5′-ATTTCAC-CTTTGTGAGTCCTACAGAAATTG-3′ and 5′-CAATTTCTGTAGGACTCACAAAGGTGAAAT-3′, 5′-CACATGGAGAAGTCAGCCCAGCGAACTGGA-3′ and 5′-TCCAGTTCGCTGGGCTGACTTCTCCAT-GTG-3′, respectively. The mismatched nucleotides for mutagenesis are underlined. pGEX-5X-1/TNNI3K, pcDNA4-Xpress/TNNI3K, and pEGFP-TNNI3K were described previously [2]. Anti-FLAG and anti-Xpress antibodies were purchased from Sigma Co. (USA) and Invitrogen Co., respectively. A matchmaker yeast two-hybrid system 3 kit and a human heart cDNA library for two-hybrid screening were purchased from Clontech Co. (USA).

Yeast two-hybrid assay. Yeast two-hybrid screening was performed using the Gal4-based matchmaker two-hybrid system 3 kit according to the manufacturer's protocol (Clontech Co.). Briefly, the cDNA fragment coding the N-terminal ANK motif of TNNI3K, TNNI3K(101-372 aa), was fused in-frame into pGBKT7 vector and then used as a bait construct to screen the pretransformed human heart cDNA library by yeast mating. Then the detailed procedures were carried out as previously described [2].

Cell culture and transfection. 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 Lipofectamine 2000 (Invitrogen Co.) 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 onto 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 diluted in blocking buffer, then probed with appropriate horseradish peroxidase-conjugated second antibodies. Blots were developed using an ECL chemiluminescent detection system (Amersham Pharmacia Biotech, USA).

Immunoprecipitation and Western blotting. Cells transiently transfected with various plasmids as indicated were lysed in cell lysis buffer (1% NP-40, 0.25% deoxycholate, 2 mM EGTA, 1 mM EDTA, 150 mM NaCl, 50 mM 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 incubating 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 GST-fused TNNI3K was produced in *Escherichia coli* using the pGEX expression system (Amersham Pharmacia Biotech). The GST-TNNI3K protein was affinity-purified on glutathione-Sepharose beads following the manufacturer's protocol but without the final elution, GST protein serving 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 AOP-1. The reactive mixture was incubated on ice for 3 h. The beads were subsequently washed in PBS, and isolated proteins were subjected to SDS-PAGE followed by immunoblot analysis using anti-Xpress antibody.

Immunocytochemistry and confocal microscopy. HEK-293FT cells were grown on glass cover slips for 24 h in DMEM supplemented with 10% fetal calf serum. Cells were then transiently cotransfected with plasmids expressing either TNNI3K-GFP or GFP along with Xpress-tagged AOP-1 as described previously. After 24 h of transfection, the cells were washed three times with PBS, followed by fixation in ice-cold acetone–alcohol (1 : 1) for 10 min. The fixed cells were washed three times in PBS prior to permeabilization in 0.2% Triton X-100 in PBS for 5 min. After being rewashed with PBS, the slides were blocked for 30 min at 37°C in blocking buffer (normal goat serum) followed by an overnight incubation at 4°C with mouse monoclonal antibody against Xpress at a dilution of 1 : 500. The samples were washed three times in PBS and then incubated with a TRITC-conjugated goat anti-mouse secondary antibody (Zhongshan Biotech, China) at room temperature for 45 min. After extensive washing, the slides were mounted and examined under a confocal-laser-scanning-microscope (Olympus Co., Japan).

***In vitro* kinase activity assay.** Cells were transfected with expression plasmids. After 48 h, lysates were prepared in lysis buffer comprising 1% NP-40, 0.25% deoxycholate, 1 mM EGTA, 1 mM EDTA, 150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 1 mM Na_3VO_4, 100 mM NaF, 1 mM β-glycerophosphate, 5 mg/ml Protease Inhibitor Cocktail Tablets (complete mini; Roche, Switzerland). 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 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 10 mM $MnCl_2$, 5 mM β-glycerophosphate, 2 mM DTT). The immunoprecipitates were incubated with a kinase reaction buffer containing 10 μCi of [γ-^{32}P]ATP and 2 μg myelin basic protein (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.

RESULTS

Identification of AOP-1 as a molecule binding to TNNI3K. To determine the function of TNNI3K, a yeast two-hybrid system was employed to search for proteins that bind to TNNI3K. Since the ANK domain is a common domain implicated in protein–protein interaction, it is reasonable to use the N-terminal ANK fragment of TNNI3K (101-372 aa) as bait. Then the human heart cDNA library was screened because TNNI3K was proved to be a cardiac-specific protein kinase. Positive clones were selected by means of a colony-lift β-galactosidase filter assay. Several clones were identified and sequenced. Computer analysis using NCBI/BLASTP revealed one such clone, the corresponding fusion protein of which was identical to the C-terminal amino acids 5-256 of human protein AOP-1, that is, just lacking the N-terminal four amino acids.

To confirm that AOP-1 did indeed bind to TNNI3K, *in vitro* binding assay was carried out. The results showed that AOP-1 protein was bound specifically by the GST-TNNI3K, while no significant binding was detected in the control experiment (Fig. 1a). Thus, consistent with the results of two-hybrid binding in yeast cells, AOP-1 interacts with TNNI3K *in vitro*.

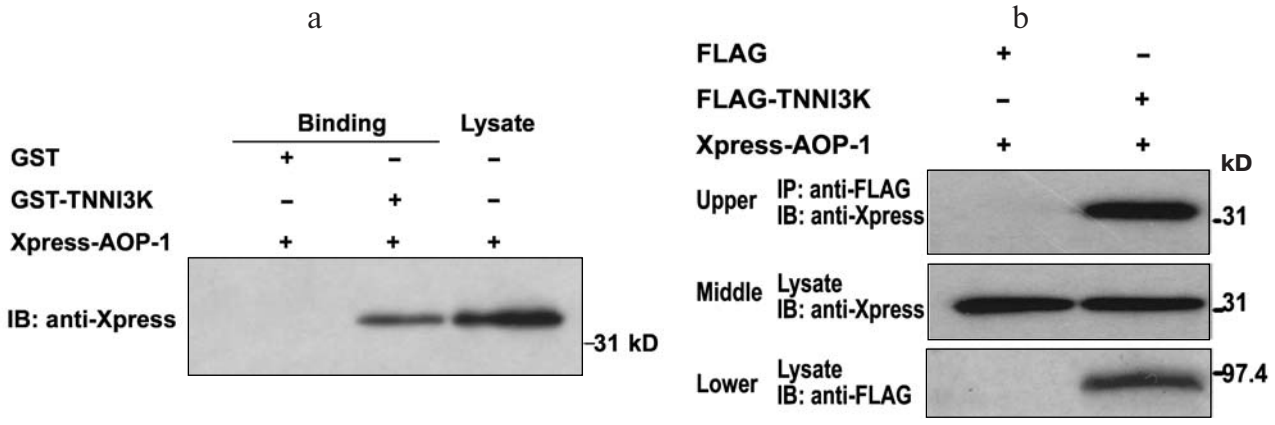

Fig. 1. Interaction between AOP-1 and TNNI3K. a) *In vitro* binding assay. The lysate prepared from HEK-293FT cells transfected with pcDNA4-Xpress/AOP-1 was equally added to GST- and GST-TNNI3K-glutathione-Sepharose beads. Proteins bound to the beads were finally analyzed by immunoblot analysis with anti-Xpress antibody. b) Xpress-AOP-1 was expressed with or without FLAG-TNNI3K in HEK-293FT cells and FLAG-TNNI3K was immunoprecipitated from each cell lysate. The presence of Xpress-AOP-1 in the immunoprecipitate was examined by Western blotting with anti-Xpress antibodies (upper). The expressions of Xpress-AOP-1 and FLAG-TNNI3K in cells were examined by immunoblotting with anti-Xpress or anti-FLAG antibodies, respectively (middle and lower).

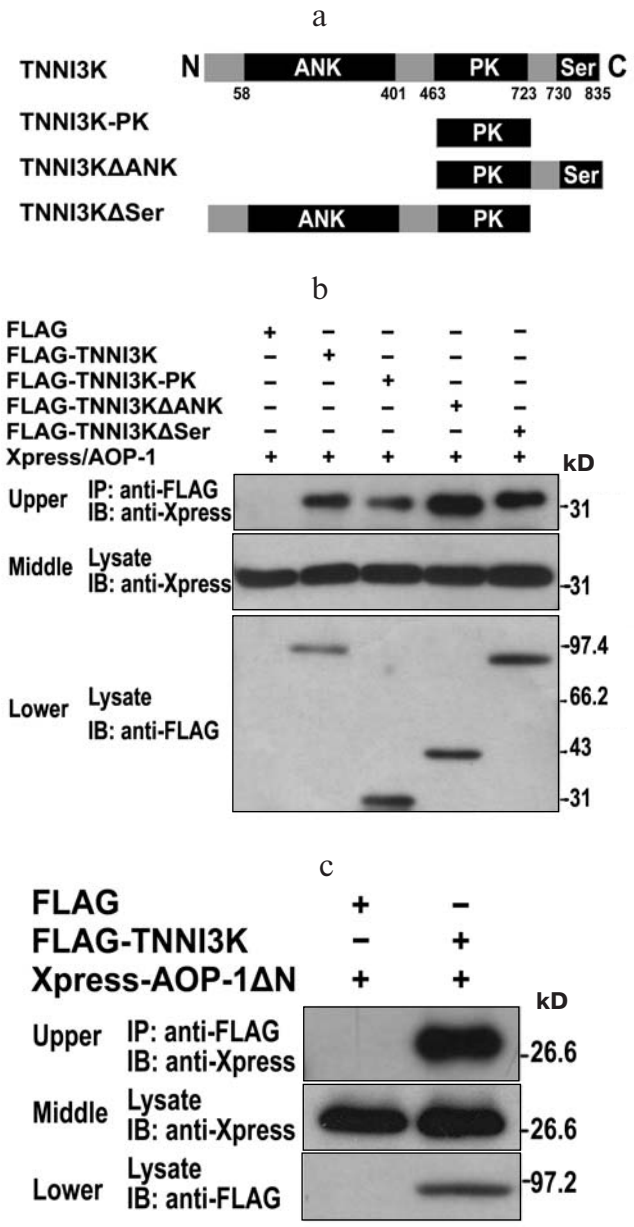

Fig. 3. Mapping of the binding domains. a) FLAG-tagged TNNI3K and its serial deletion mutants used in this study are schematically represented. b) Mapping of the AOP-1 binding sites in the TNNI3K. FLAG-tagged TNNI3K and its serial mutants were co-immunoprecipitated with Xpress-tagged AOP-1 respectively (upper). The expressions of Xpress-AOP-1 and TNNI3K serial mutants were examined by immunoblotting with anti-Xpress or anti-FLAG antibodies, respectively (middle and lower). c) Mapping of the TNNI3K binding sites in the AOP-1. Xpress-AOP-1ΔN was expressed with or without FLAG-TNNI3K in HEK-293FT cells, followed by immunoprecipitation with anti-FLAG antibody (upper). The amounts of Xpress-AOP-1ΔN (middle) and FLAG-TNNI3K (lower) in each cell lysate were determined as above.

Next, whether AOP-1 could associate with TNNI3K or not was tested in mammalian cells. As shown in Fig. 1b, the Xpress-tagged AOP-1 was co-precipitated with FLAG-tagged TNNI3K, but not with control FLAG alone. To exclude the possibility of the interaction induced by these two kinds of tags, an unrelated protein cardiac troponin T (cTnT) tagged with Xpress was used to do the same co-immunoprecipitation assay with FLAG-tagged TNNI3K. The result showed that Xpress-tagged cTnT did not co-precipitate with FLAG-tagged TNNI3K (data not shown), which demonstrated that there was no interaction between Xpress and FLAG. Thus, the co-immunoprecipitation of Xpress-tagged AOP-1 with FLAG-tagged TNNI3K was actually due to the interaction between AOP-1 and TNNI3K *in vivo*.

Colocalization of AOP-1 and TNNI3K. To examine the subcellular localization of the AOP-1 and TNNI3K, HEK-293FT cells were cotransfected with expression plasmids encoding Xpress-tagged AOP-1 and either green fluorescent protein (GFP)–TNNI3K fusion protein. Figure 2 (see color insert) shows that Xpress-tagged AOP-1 (red fluorescence) was exclusively expressed in the cytoplasm. At the same time, green fluorescence indicated the GFP–TNNI3K, which also appeared diffusely distributed throughout the cytoplasm, though it was distinguished with the previous finding [2]. As shown in the right panel, there was colocalization of AOP-1 and TNNI3K in yellow. The data further suggest that AOP-1 could interact with TNNI3K *in vivo*.

Mapping of the binding domains. After AOP-1 was identified as a specific binding protein for full-length TNNI3K, the molecular sites involved in the AOP-1–TNNI3K interaction were sought to be defined, as a series of deletion mutants of TNNI3K had been constructed [4]. Then the co-immunoprecipitation was carried out again. As shown in Fig. 3b, the serial TNNI3K deletion mutants, each containing the protein kinase domain, were all co-immunoprecipitated with AOP-1. This suggested that the kinase domain in addition to the ANK motif of TNNI3K were responsible for the interaction with AOP-1, as the bait protein used for the yeast two-hybrid screening just consisted of the ANK domain of TNNI3K. An expression vector of AOP-1 mutant, which lacks the N-terminal mitochondrial targeting signal region (1-60 amino acids) of AOP-1 (AOP-1ΔN), was also constructed [3]. The test of co-immunoprecipitation showed that it could also interact with the full-length TNNI3K (Fig. 3c). Thus, the binding site was located within the C-terminal 60-256 amino acids of AOP-1, that is, the thiol-specific antioxidant domain.

Antioxidant activity of AOP-1 is not essential for its association with TNNI3K. The AOP-1 belongs to an expanding family of thiol-specific antioxidant proteins [5, 6]. It has two cysteine residues (C108 and C229) that are conserved in thioredoxin-dependent peroxidases and essential for the antioxidant activity [3, 5]. AOP-1 is believed to form homodimers within cells. When AOP-1 reduces reactive oxygen species, an intermolecular disulfide bond is formed between the two conserved cysteine residues of AOP-1 dimers [5, 7]. Since TNNI3K bound

to the antioxidant domain of AOP-1, we next determine whether these conserved cysteine residues are necessary for AOP-1 to bind to TNNI3K. Two point mutants, in which one of the two conserved cysteine residues was mutated to a serine residue (AOP-1 C108S and C229S, respectively), were constructed. As shown in Fig. 4, both AOP-1 C108S and C229S were co-immunoprecipitated with TNNI3K, indicating that these conserved cysteines are not essential for the binding of AOP-1 to TNNI3K.

AOP-1 inhibited the kinase activity of TNNI3K. To determine whether AOP-1 has a biochemical effect on TNNI3K, an *in vitro* kinase assay was carried out. In the series of deletion mutants of TNNI3K, only TNNI3KΔSer, lacking the C-terminal Ser-rich domain and exhibiting negative regulation of the kinase [4], had the kinase activity, so it was taken into account at the same time. HEK-293FT cells were co-transfected with Xpress-tagged AOP-1 and FLAG-TNNI3K or FLAG-TNNI3KΔSer, respectively, followed by an immune complex-coupled kinase assay using MBP as substrate. After co-expressed with AOP-1, the amounts of autophosphorylated TNNI3K as well as that of phosphorylated MBP were decreased a little compared with the control (Fig. 5). As for TNNI3KΔSer, the inhibition of the autophosphorylation and the phosphorylation of substrate were much more significant after co-expression of AOP-1. These data indicated that AOP-1 could down-regulate the kinase activity of TNNI3K.

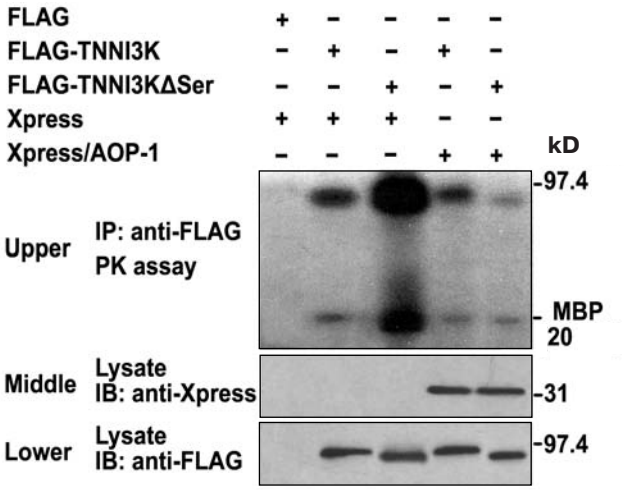

Fig. 5. Inhibition of the kinase activity of TNNI3K by co-expression of AOP-1. HEK-293FT cells were transiently co-transfected with FLAG-tagged TNNI3K expression plasmid (pcDNA6-FLAG/TNNI3K) or FLAG-tagged TNNI3KΔSer expression vector with or without the expression construct of Xpress-tagged AOP-1. Then proteins were immunoprecipitated with anti-FLAG antibody. The immune complex was incubated with the substrate MBP and [γ-^{32}P]ATP, and then kinase activity was measured by autoradiography (upper). The amounts of Xpress-AOP-1 (middle) and FLAG-TNNI3K or FLAG-TNNI3KΔSer (lower) were determined as above.

DISCUSSION

Our previous study showed that TNNI3K is a functional kinase. In order to get the biological function(s) clues of TNNI3K, the interaction partners that might serve as regulators or substrates or downstream effectors were sought. The yeast two-hybrid screen revealed AOP-1, which was originally reported to be a mitochondrial antioxidant protein, and additional proteins that were not analyzed in the present study. The specific interaction between TNNI3K and AOP-1 was confirmed by *in vitro* binding assays and coexpression experiments. Furthermore, coexpression of AOP-1 and TNNI3K resulted in the decreasing of the kinase activity of TNNI3K. Our data identify AOP-1 as a new modulator of TNNI3K kinase activity, and we speculate that TNNI3K may thus participate in various biological processes that AOP-1 is associated with in the cardiomyocytes.

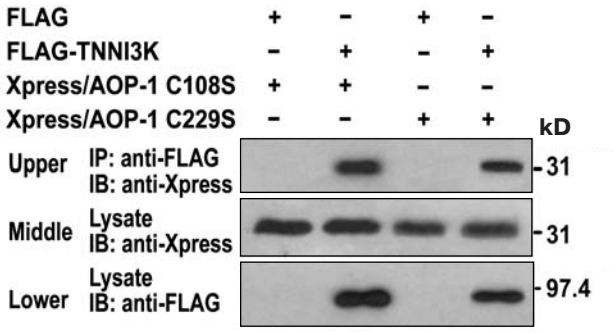

Fig. 4. Antioxidant activity of AOP-1 is not essential for binding to TNNI3K. Xpress-tagged AOP-1 mutants were co-expressed with or without FLAG-TNNI3K in HEK-293FT cells. After immunoprecipitation of TNNI3K with anti-FLAG Ig, the presence of AOP-1 mutants in the immunoprecipitate was examined by Western blotting with anti-Xpress Ig (upper). The expressions of Xpress-tagged AOP-1 mutants (middle) and FLAG-TNNI3K (lower) in each cell lysate were examined as described above.

In this study, after transient transfection, GFP-TNNI3K was observed to be predominantly expressed in the cytoplasm of HEK-293FT cells and as well as the COS-7 cells (data not shown) under the confocal microscope, which was distinct with the previously demonstrated nucleus-localization of TNNI3K by immunohistochemistry with polyclonal antibody produced by a vaccine-based method [2]. To make it clear, we produced the specific monoclonal antibody against TNNI3K, followed by the immunostaining of the adult heart samples. Consistent with the results of ectopic expression, TNNI3K was shown to distribute significantly in the cytoplasm of cardiac myocytes (data not shown).

The highly specific interaction of TNNI3K–AOP-1 has been consistently observed under a variety of experimental conditions. In addition to interacting in yeast cells, the purified TNNI3K recombinant proteins and

AOP-1 fusion proteins readily bind to each other *in vitro*. Furthermore, native TNNI3K and AOP-1 were co-immunoprecipitated from mammalian cells, suggesting that they could also form a multiprotein complex in cells. Finally, partial cytoplasmically expressed TNNI3K proteins are colocalized with AOP-1, consistent with the conclusion.

AOP-1 was firstly identified as a molecule that exhibits sequence similarity to mouse MER5, which is localized in mitochondria [8]. AOP-1 is also localized in mitochondria [3]. However, in this study, AOP-1 expression was not confined to mitochondria but diffused in cytoplasm. Meanwhile, TNNI3K was also shown to be localized in the cytoplasm as we demonstrated above. Recently, the associations of overexpressed AOP-1 with other cytosolic proteins were also reported [3, 5, 9]. Thus, cytosolic distribution of AOP-1 might be due to the overexpression. In turn, it got the chance to interact with these cytosolic proteins. On the other hand, it is well known that mitochondrial proteins are present in the cytoplasm under apoptotic conditions. Therefore, it is possible that TNNI3K associates with AOP-1 under some pathogenic conditions, such as apoptosis.

TNNI3K interacting proteins could be its regulators or substrates. Then, we performed the *in vitro* kinase assay to clarify it. The result revealed that AOP-1 was a negative regulator of TNNI3K. As our data demonstrated that AOP-1 could not only bind to the ANK motif but also the protein kinase domain of TNNI3K, it is reasonable to propose that AOP-1 performed its inhibition through its association with the protein kinase domain of TNNI3K, which in turn interfered with substrate binding to this domain. However, the exact mechanism remains to be determined.

AOP-1, a member of a newly discovered family of peroxidases (peroxiredoxins), efficiently reduced the intracellular level of reactive oxygen species (ROS) such as H_2O_2 in the presence of thioredoxin. Among the members of peroxiredoxins, AOP-1 is the only one located in mitochondria [3, 5], which is the major site of cellular ROS generation. It can scavenge ROS by cooperating with mitochondrial thioredoxin and can protect mitochondrial components from the action of superoxide anions or hydrogen peroxide [3]. Results using mitochondria-specific fluorescent probes demonstrated that AOP-1 is essential to maintain mitochondrial mass and membrane potential [10]. Araki et al. confirmed that AOP-1 plays a crucial role in the antioxidant defense mechanism of mitochondria in the cardiovascular system, using cultured bovine aortic endothelial cells and an *in vivo* model of experimental myocardial infarction [11]. In addition to its role as a peroxidase, however, a body of evidence has accumulated to suggest that AOP-1 also associated with various biological processes such as cell proliferation, differentiation, apoptosis, and gene expression [6]. The mouse AOP-1 protein, also called Mer5, may promote early events in the differentiation of murine erythroleukemia cells [12]. AOP-1 was overexpressed in hepatocellular carcinoma and breast cancer tissues compared to adjacent normal tissues [13-15]. Wonsey et al. showed that AOP-1 was a Myc target gene and it was required for Myc-mediated proliferation, transformation, and apoptosis [10]. Whether or not these biological functions are due to its regulation of the intracellular level of signaling molecules, ROS, remains to be determined.

Since our data revealed that AOP-1 specifically bound to TNNI3K and down-regulated its kinase activity, we propose that TNNI3K participates in the signal transduction of various pathophysiological processes that AOP-1 is involved in in cardiomyocytes, such as oxidative stress, apoptosis, and so on. Future studies will focus on the exact role of the interaction.

This work was supported by grants to Xianmin Meng from the National Natural Science Foundation of China (No. 30470724), to Laiyuan Wang from the National Natural Science Foundation of China (No. 30500200), and to Yan Feng from the Science and Technique Development Foundation of Tongji University (No. 1509219021).

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