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14-3-3ζ interacts with human thromboxane receptors and is involved in the agonist-induced activation of the extracellular-signal-regulated kinase

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ARTICLE INFO

Article history:
Received 4 October 2005
Accepted 28 November 2005

Keywords:
14-3-3ζ
Thromboxane receptor
Raf-1
Signal transduction
ERK

Abbreviations:
BLAST, basic local alignment search tool
ct, carboxyl terminal tail
DMEM, Dulbecco modified Eagle's medium
EGFR, epidermal growth factor receptor
ERK, extracellular-signal-regulated kinase
FBS, fetal bovine serum
GAPDH, glyceraldehyde-3-phosphate dehydrogenase
GPCR, G-protein-coupled receptor
GSH, glutathione
GST, glutathione S-transferase
HRP, horseradish perioxidase
i3, intracellular loop 3

ABSTRACT

Thromboxane receptor (TP) signaling results in a broad range of cellular responses including kinase activation and subsequent nuclear signaling events involved in cell transformation, proliferation, and cell survival. Proteins that may participate in the early signaling following receptor activation remain to be identified. We found that 14-3-3ζ is a novel protein interacting with TP intracellular loop 3 (i3) by yeast two-hybrid system. This interaction was further confirmed by GST pull-down and co-immunoprecipitation methods. Site-directed mutagenesis studies indicated that Pro-236 of the TP-i3 was involved in the binding to the 14-3-3ζ. Co-immunoprecipitation studies in the same cell lysate by TP antibody showed that TP binds not only with the 14-3-3ζ but also with the Raf-1. Our data also demonstrated that TP receptor activation induced by agonist rapidly recruited 14-3-3ζ and Raf-1 to form a complex with the TP on the plasma membrane. The significance of assembling this protein complex was examined by TP agonist-induced extracellular-signal-regulated kinase (ERK) phosphorylation in intact cells. TP agonist, I-BOP, induced ERK phosphorylation in HEK 293 cells expressing wild type TPα but significantly lower in those expressing TPα-P236V mutant. Attenuation of the expression of 14-3-3ζ by 14-3-3ζ siRNA decreased I-BOP-induced ERK phosphorylation indicating the involvement of the 14-3-3ζ in the signal transduction process. These results suggest that 14-3-3ζ may serve as a scaffold protein to form a protein complex consisting of TP, 14-3-3ζ, and Raf-1, and that this protein complex may be involved in the activation of ERK pathway following TP receptor activation.

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0006-2952/$ – see front matter
doi:10.1016/j.bcp.2005.11.027

I-BOP, [1S-α,2α(Z),3β(1E,3S),4α]]-7-[3-[3-hydroxy-4-(4-iodophenoxy)-1-butenyl]-7-oxabicyclo[2,2,1]hept-2-yl]-5-heptenoic acid
IP, immunoprecipitation
MAPK, mitogen-activated protein kinases
15-PGDH, 15-hydroxyprostaglandin dehydrogenase
PMSF, phenylmethylsulfonyl fluoride
PVDF, polyvinylidene fluoride
SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TXA_2, thromboxane A2
TPα/β, thromboxane A2 receptor α or β form

1. Introduction

Thromboxane A_2 (TXA_2)[1] is a biologically active eicosanoid primarily released from activated platelets, monocytes, and some cancer cells [1]. It is a product of the sequential metabolism of arachidonic acid by cyclo-oxygenases (COXs) and thromboxane synthase [2]. It induces platelet aggregation and secretion, stimulates vaso- and broncho-constriction, and has been implicated in a number of cardiovascular and bronchial diseases [3]. In addition, its mimetic stimulates mitogenesis and the hypertrophy of vascular smooth muscle cells. These actions are believed to be mediated by cell surface thromboxane receptors (TPs), which belong to a G-protein-coupled receptor superfamily. Two isoforms, derived from alternate splicing of carboxyl-terminal tail of the receptor, have been cloned [4]. Human TPα mRNA encoded a protein of 343 amino acids, whereas TPβ mRNA encoded a protein of 406 amino acids and is identical in sequence to TPα mRNA for the first 328 amino acids. The only structural difference of these two isoforms resides in the cytoplasmic C-terminal tails.

Mitogenic and hypertrophic effect, and activation of mitogen-activated protein kinases (MAPKs) induced by TP agonists have been reported in several cell lines. TP agonists were shown to trigger a protein kinase C (PKC), Src, and epidermal growth factor receptor (EGFR)-dependent activation of extracellular-signal-regulated kinase (ERK) in a pertussis toxin sensitive manner in human endothelial cell line, ECV 304 [5]. U-46619, one agonist of TPs, was also demonstrated to induce a PKC, PKA, phosphoinositide (PI) 3-kinase, and EGFR-dependent activation of ERK in a pertussis toxin independent manner in human uterine smooth muscle cell line, ULTR [6,7]. U-46619 was also reported to induce C-Jun N-terminal kinase (JNK) activation and cyclo-oxygenase-2 expression in a PKA-dependent manner in porcine aortic smooth muscle cells [8]. Recently, Gallet et al. [9] demonstrated that matrix metalloproteinases mediated partially TP agonist-dependent transactivation of EGFR and subsequent activation of ERK in human aortic smooth muscle cells. The signal transduction pathways of TPs leading to ERK activation appear to be rather complex and remain to be further elucidated.

14-3-3 proteins were first identified as highly abundant acidic proteins in mammalian brain extracts and successively demonstrated in other tissues. So far, there are seven isoforms cloned [10]. All of the 14-3-3 proteins form homodimers and/or heterodimers that interact with signaling and other functional proteins [11] and play important roles in various cellular processes such as signal transduction, cell cycle regulation, apoptosis, stress response, cytoskeleton organism, and malignant transformation [12–14]. These proteins are essential for regulated signal transduction and may not directly activate or inhibit kinases, but rather behave as a ''scaffold'' or ''anchor'' to localize protein kinase activity. It has been well established that the activation of ERK is dependent on the activation of Raf-1 which binds to 14-3-3ζ [15]. Recently, Hekman et al. [16] reported the dynamic changes in Raf-1 phosphorylation and the 14-3-3ζ binding. It was proposed that the 14-3-3ζ had differential roles in the different steps of the activation of the Raf-1 in response to the growth factor stimulation.

In the present study, we found that TPs bound to the 14-3-3ζ through their intracellular loop 3 (i3) domain and Raf-1 was also in the protein complex with TPs. After stimulation by a TP agonist, the protein complex of the 14-3-3ζ and the Raf-1 were recruited to the cytoplasmic side the membrane to interact with TPα and then Raf-1 was activated leading to the eventual phosphorylation of ERK. We discovered that the blockade of the interaction between the TPs and the 14-3-3ζ decreased agonist-induced activation of the ERK.

2. Materials and methods

2.1. Materials

Culture medium, heat-inactivated fetal bovine serum (FBS) and restriction enzymes were from Invitrogen or Gibco (Carlsbad, CA). I-BOP was from Cayman Chemical (Ann Arbor,

MI). The other biochemicals and chemicals were obtained from Sigma (St. Louis, MO). Antibodies specific for pERK and 14-3-3ζ, 14-3-3ζ siRNA (pooled sequences: #1, GGUACAUUGUGGCUUCAAATT; #2, GCUUCCAUGUCUAAGCAAATT; #3, CCAGUCACAGGUGUAGUAATT; #4, GGUUCUGAAACUUCUCUAATT) and control siRNA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody specific for ERK (p42 and p44) were purchased from Cell Signaling Technology (Beverly, MA). Antibody specific for Raf-1 was from Lab Vision Corp (Fremont, CA). Antibodies specific for thromboxane receptor (TP) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were generated in house [17,18]. Horseradish peroxidase (HRP)-linked goat anti mouse and rabbit IgG were supplied by Transduction Laboratories (Lexington, KY). The HEK 293 cell lines were supplied by the American Type Culture Collection (Manassas, VA). *DupLEX-A*™ yeast two-hybrid system and human prostate tumor cDNA library encoded by the yeast two-hybrid vector pJG4–5 were provided by Origene Technologies (Rockville, MD). All the plasmids used in the yeast two-hybrid system were obtained from Origene Technologies. ECL Western blotting detection system and the plasmid of pGEX-2T for expressing GST fusion proteins were supplied by the Amersham Pharmacia Biotech (Cardiff, UK). The plasmid encoding human TPα cDNA in pcDNA3 vector was obtained as reported previously [19].

2.2. Construction of bait vectors

To create the ''bait'' vectors, the TPα/β intracellular loop 3 (TPα/β-i3) domain was cloned downstream of the DNA binding domain of Gal4, in the plasmid of pEG202. The coding sequence of 15-PGDH was cloned into plasmid pEG202 as a control bait to test non-specific binding between the bait and the prey.

2.3. Yeast two-hybrid system screen

Growth and transformation of yeast strain EGY48 were performed as described in the manual of the *DupLEX-A*™ yeast two-hybrid system. Briefly, a human prostate tumor cDNA library encoded by the yeast two-hybrid vector pJG4–5 was transformed into the yeast strain EGY48 that was previously co-transformed by pEG202-TPα/β-i3 bait vectors and pSH18–34 reporter gene vector. The transformed cells were then analyzed by growth on drop-out medium lacking tryptophan, leucine, histidine, and uracil and grown for 4 days at 30 °C. The positive colonies were confirmed using β-galactosidase assay. After the second round of screening performed with a pRHFM1 plasmid as a negative control and pEG202-15-PGDH bait as a non-specific control, library plasmid cDNAs were rescued, sequenced, and identified using the BLAST algorithm.

2.4. Expressions of intracellular loop 3 (i3) and C-terminal tail (ct) of the TPα and 14-3-3ζ as glutathione S-transferase (GST)-fusion proteins

The DNA sequences encoding the i3 (ATLCHVYHGQEAAQQRPRDSEVEMMAQ) and the ct (RRAVLRRLQPRLSTRPRSLSLQPQLTQRSGLQ) of TPs and 14-3-3ζ were generated by PCR using *Taq* polymerase. PCR amplification was carried out in 50 μl of final volume, with 0.2 mM dNTP, 100 ng of each primer, 100 ng of template, and 1.25 units *Taq* DNA polymerase under the conditions of 94 °C, 1 min, 60 °C, 1 min, 72 °C, 1 min for 30 cycles. The digested PCR products were ligated into *Bam*HI and *Eco*RI sites of the pGEX-2T GST fusion proteins expression vector. The cloning junctions were sequenced using pGEX sequencing primer (5′-GGGCTGGC AAGCCACGTTT GG-3′) to verify that inserts were in-frame with the GST gene. The recombinant pGEX-2T plasmid DNA was purified and transformed into *E. coli* BL21 for bacterial expression. The GST fusion proteins were purified using GSH-agarose affinity chromatography as previously described [20].

2.5. Membrane overlay experiment

Ten micrograms of GST and GST fusion proteins of TP-i3 and TPα-ct were resolved in 12% SDS-PAGE gel, following the electrophoretically transference to PVDF membrane. The membrane was incubated with 1 μg/ml GST-14-3-3ζ in 30 mM Tris–HCl, pH 7.4, 120 mM NaCl (TBS) buffer containing 5% non-fat milk for 2 h at the room temperature. Then the membrane was probed with 14-3-3ζ antibody by the procedure of Western blotting as indicated below.

2.6. GST pull-down assay

HEK293 cells were lysed in ice-cold lysis buffer (1% Nonidet P-40 in 150 mM NaCl, 50 mM HEPES, pH 7.4, 5 mM NaF, 5 mM pyrophosphate, 1 mM sodium orthovanadate, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM PMSF) for 1 h on ice. The lysates were spun down at 15,000 × *g* for 20 min at 4 °C. The 50 μg total protein of the cleared lysates was incubated with 5 μg of the GST or GST fusion proteins and GSH-agarose beads for 4 h at 4 °C. The GSH-agarose was precipitated by centrifugation, washed three times with lysis buffer, treated with SDS-PAGE loading buffer, boiled, and subjected to 12% SDS-PAGE gel. The bound proteins were electrophoretically transferred onto PVDF membrane and detected by Western blotting as indicated below.

2.7. Site-directed mutagenesis

All site-directed mutagenesis of TPα and TPα/β-i3 cDNA was performed by QuikChange site-directed mutagenesis method [21]. Two pairs of PCR primers were used to perform PCR to generate P236V and S239D mutants. The template is either pCDNA3-TPα or pGEX2T-TPα/β-i3. The PCR product was treated with *Dpn*I endonuclease to digest the parental DNA template and transformed into XLI-Blue competent cells. After DNA sequences of the mutants were confirmed by DNA sequencing, pGEX2T constructs were transformed into *E. coli* Bl21 for bacterial expression and the pCDNA3 constructs were used for transient and stable transfection of the mammalian cells.

2.8. Cell culture and transfection of TP constructs and siRNA

HEK293 cells were cultured as monolayer in DMEM supplemented with 10% heat inactivated FBS, gentamicin, and antibiotic–antimycotic at 37 °C in a humidified atmosphere of

95% air and 5% CO_2. The cells were subcultured in 6 well plates or 12 well plates to achieve 80% confluence on the next day, and then the cells were transfected with pCDNA3-TPα or its mutants using lipofectamine reagent. For transient transfection, the cells were allowed to grow in the 10% FBS medium for 24 h, followed by starvation in FBS free medium for 16 h to introduce quiescence. The cells then were stimulated by a TP agonist. For stable transfection, the cells were allowed to grow in 10% FBS medium for 48 h after transfection and then the cells were treated with 1 mg/ml G418 until the colonies appear. The 14-3-3ζ siRNA at the concentration of 20 pmol/well in 12 well plates was introduced to HEK293-TPα cells by lipofectamine reagent. The cells were treated with the siRNA for 48 h to achieve the maximal blockade of the expression of the 14-3-3ζ.

2.9. Co-immunoprecipitation of the 14-3-3ζ and the Raf-1 by TP antibody

The cell lysate of HEK293 cells stably transfected with TPα was incubated with TP antibody or control antibody with shaking for 3 h at 4 °C, and then protein A agarose beads were added and continuously incubated with shaking for another 3 h. The beads were collected by centrifugation, washed six times with lysis buffer, treated with SDS-PAGE loading buffer, boiled, and subjected to 12% SDS-PAGE gel. The presence of 14-3-3ζ and Raf-1 was detected respectively by Western blot as indicated below.

2.10. Western blot analysis

The cells were lysed in lysis buffer for 1 h on ice. Cleared lysate was then subjected to 12% SDS-PAGE gel. Proteins were then electrophoretically transferred onto PVDF membrane. The membrane was blocked with 5% non-fat milk in 30 mM Tris–HCl, pH 7.4, containing 120 mM NaCl (TBS) at room temperature for 1 h. It was then incubated for 2 h at room temperature with a primary antibody in TBS with 5% non-fat milk and then the membrane was washed three times with TBS buffer containing 0.05% Tween-20 (TBST), following incubation with HRP-linked goat anti mouse or rabbit IgG for 1 h at room temperature. At last the membrane was washed with TBST for three times. The immunoreactive bands were detected using ECL Western blotting detection system.

3. Results

It is well known that intracellular loops and C-tail of the G-protein-coupled receptors are involved in signal transduction traffics. In an attempt to search for TP-associated proteins, the yeast two-hybrid system was used to identify proteins that might interact with TP-i3. After screening 5×10^6 clones from a human prostate tumor cDNA library using the TP-i3 bait, several potential partners were identified based on the growth on the -leu plates and β-galactosidase assay. The potential positives were chosen for a second round confirmation by co-transforming their plasmids rescued from the yeast with original bait and reporter vector. The non-specific bait pEG202-15-PGDH and negative control vector PRHFM-1 were also co-transformed with the potential positives and reporter vector. We identified one clone (clone 5) which had a very strong interaction with the TP-i3 but no interaction with non-specific bait and negative control (Table 1). After rescuing the plasmid DNA from that clone, it was digested with *Xho*I and *Eco*R1, and yielded a 0.5 kbp pair cDNA fragment. This fragment was sequenced and analyzed in the nucleotide database by the BLAST program. It was found that this fragment encodes the first 0.5 kbp from the N-terminal end of the coding sequence (0.735 bp) of the 14-3-3ζ.

In order to test the in vitro binding of the 14-3-3ζ to the TP-i3, we constructed GST fusion proteins of the 14-3-3ζ and the TP-i3. We also constructed the GST-TPα-ct to see if the 14-3-3ζ binds to the C-tail of the TPα. The membrane overlay experiment showed that GST-TP-i3, which was electrophoresed and transferred onto the PVDF membrane, can bind and accumulate GST-14-3-3ζ (Fig. 1a, top panel). However, GST-TPα-ct and GST alone cannot interact with the GST-14-3-3ζ on the membrane. These results demonstrated that there were no GST dimerizations in this condition, because the binding was performed in denatured condition after the first GST and GST fusion proteins were subjected to SDS-PAGE. Therefore, these data showed the specific binding between the native 14-3-3ζ and denatured TP-i3, indicating that only several amino acids in the primary structure of the TP-i3 were involved in the binding. In order to control the loading amount of the protein on the membrane, same amount of three different proteins were resolved by the SDS-PAGE and stained by Coomassie Brilliant Blue (Fig. 1a, bottom panel).

In order to identify the key amino acids involved in the binding between the TP-i3 and the 14-3-3ζ, site-directed mutagenesis was performed on TP-i3. We examined the TP-i3 sequence (27 amino acids) and found that there is no 14-3-3ζ binding consensus by using Scansite website (http://scansite.mit.edu). However, when we performed the alignment between the TPα and the α2-adrenergic receptor, including three subtypes, 2A, 2B, and 2C, which were the first identified GPCRs interacting with 14-3-3ζ through the intracellular loop 3 domain [22], the Pro-236 of the TP was found as a conserved

Table 1 – The results of the second round confirmation of the interaction between the TPα/β-i3 and the clone 5

Baits	TPα/β-i3	pRFHM-1[a]	15-PGDH[b]
Growth on YNB(gal)-Leu-Ura-His-Trp[c]	+++	–	–
β-Galactosidase activity[d]	+++	+	–

[a] Negative control plasmid provided by the vendor.
[b] 15-PGDH constructed as a control bait to test the non-specific binding.
[c] +++, excellent growth; ++, some growth; +, poor growth; –, no growth.
[d] +++, dark blue; ++, blue; + weak blue; –, white.

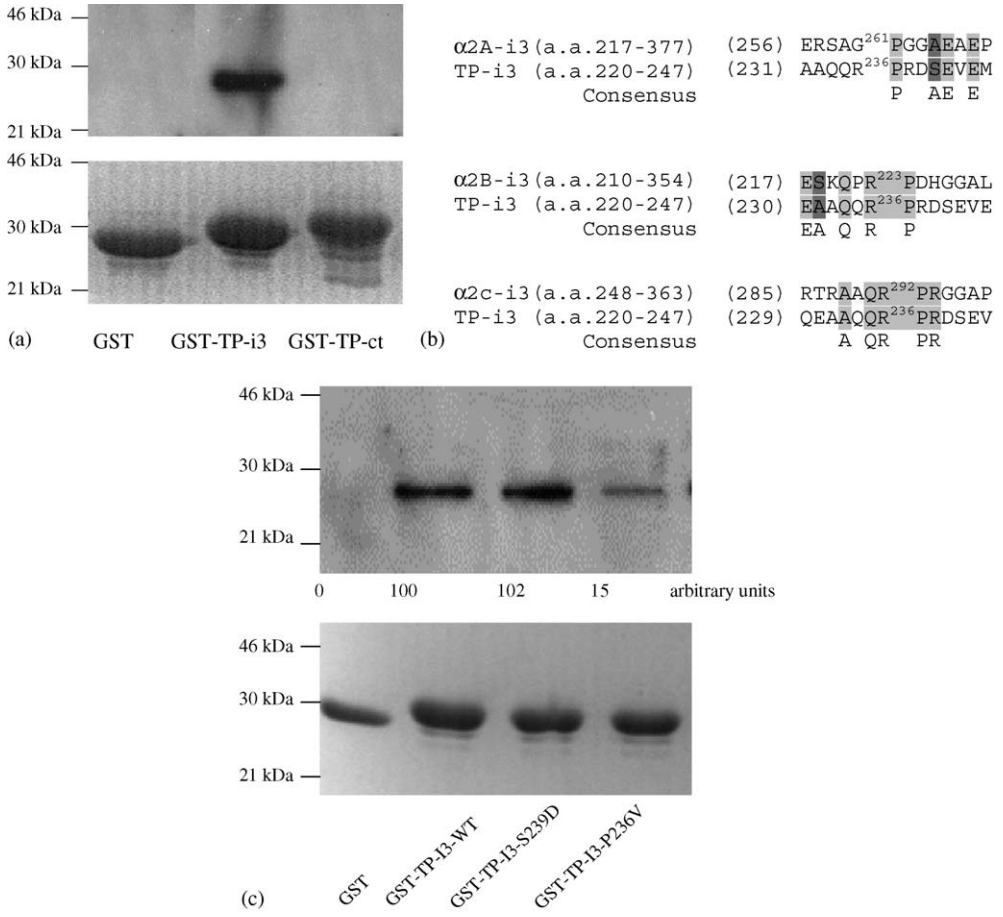

Fig. 1 – 14-3-3ζ interacts with TP-i3 in vitro. (a) 14-3-3ζ interacts specifically with the i3 domain of the TPs in vitro by membrane overlay experiments. GST-14-3-3ζ, GST-TPα/β-i3, GST-TPα-ct, and GST alone proteins were expressed and purified from *E. coli* as described in Section 2. Ten micrograms of GST-TPα/β-i3, GST-TPα-ct, and GST are subjected to 12% SDS-PAGE gel, following transferring to PVDF membrane. One micrograms per milliliters GST-14-3-3ζ in TBS containing 5% non-fat milk was incubated with the membrane following detection of 14-3-3ζ by polyclonal antibody as described in Section 2 (top panel). The same amount of GST and GST fusion proteins were Coomassie Blue-stained and were aligned to show the loading control (bottom panel). Positions of molecular weight standards were shown at the left. (b) Part of alignment of TP-i3 sequence with i3 domain of α2-adrenergic receptor subtypes 2A, 2B, and 2C, generated by Vector NTI software. (c) Pro-236 of the TP is essential for TP/14-3-3ζ interaction in vitro. Five micrograms of bacterially produced GST-TP-i3 proteins (wild type and P236V and S239D mutants) were used to pull-down the 14-3-3ζ from the HEK293 cell lysates as described in Section 2, and the 14-3-3ζ was detected by Western blotting analysis (top panel). The Coomassie Blue-stained GST and GST fusion proteins were used to ensure the same amount of proteins loaded in the pull-down experiments (bottom panel). The 14-3-3ζ bands densities were normalized to the GST fusion protein loaded were presented by arbitrary units, in which the band of wild type was set to 100. Results are representative of three independent experiments.

residue in all these receptors (Fig. 1b). It has been shown that the serine phosphorylation within the motif is essential to the 14-3-3ζ binding (23, 24). Therefore, mutants of TP-i3-P236V and TP-i3-S239D were constructed as GST fusion proteins and purified as described [20]. The mutation of Ser-239 to an aspartic residue is to generate a negative charge much like a serine residue following phosphorylation. GST fusion proteins were used to pull-down the 14-3-3ζ from the HEK293 cell lysate. The results of GST pull-down experiment showed that GST-TP-i3 wild type and GST-TP-i3-S239D mutant could pull-down the same amount of the 14-3-3ζ from the HEK293 cell lysate as detected by the 14-3-3ζ antibody in the Western blot (Fig. 1c), indicating that the charge on the residue at Ser-239 position may not be essential for interaction. However, the GST-TP-i3-P236V mutant showed attenuated ability to pull-down the 14-3-3ζ and the GST could not pull-down the 14-3-3ζ at all from the HEK293 cell lysate. These results demonstrated that Pro-236 in TP-i3 was directly involved in the interaction with 14-3-3ζ.

The 14-3-3ζ is known to be a scaffold protein that binds more than one protein [11]. It is very likely that the 14-3-3ζ may also interact and form a protein complex with other proteins in addition to the TPs. So far, the 14-3-3ζ has been reported to interact with PI-3-kinase [25], PKC [26], and Raf-1 [27] which

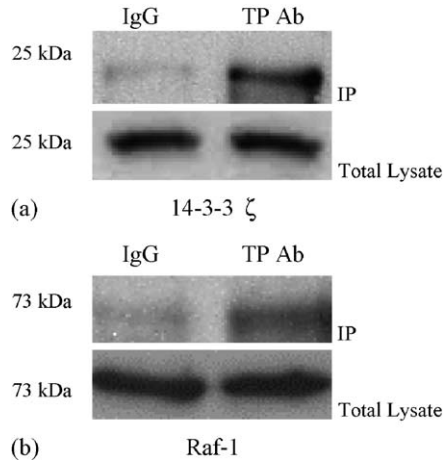

Fig. 2 – 14-3-3ζ and Raf-1 were co-immunprecipitated with TPα in vivo. After 50 nM I-BOP stimulation for 10 min, the lysates from TPα stably transfected HEK293 cells were prepared as described under Section 2. The TP co-immunoprecipitation was performed using TP N-terminal polyclonal antibody, following absorption by protein A agarose beads. The precipitate was subjected to SDS-PAGE and analyzed by Western blotting. The 14-3-3ζ (a) and Raf-1 (b) bands were detected by their specific polyclonal antibodies. As a negative control, against normal rabbit antibody was used for immunoprecipitation (IgG panel in both a and b). IP: immunoprecipitation; total lysate: the whole cell lysates used to perform the immunoprecipitation. Results are representative of three independent experiments.

was shown to be involved in the I-BOP-induced activation of ERK. Therefore, immunoprecipitation was performed to identify the components of the receptor complex of the TPs. Preliminary study indicated that TP interacted with the maximal amount of the 14-3-3ζ at 10 min following stimulation by I-BOP, one of agonists of TPs, in TP alpha stably transfected HEK293 cells. Therefore, the immunoprecipitation by TP antibody was conducted at 10 min following stimulation by the agonist. The Raf-1 was identified as a protein in the receptor complex in addition to the 14-3-3ζ (Fig. 2).

The immunoprecipitation was then performed to monitor the dynamic binding between the 14-3-3ζ or the Raf-1 and the TPα following stimulation by I-BOP. The TP antibody immunoprecipiated some basal level of the 14-3-3ζ without stimulation by I-BOP as shown in Fig. 3a. The amount of the immunoprecipated 14-3-3ζ began to increase at 2 min and continued to increase within the first 10 min of agonist stimulation and then began to decrease in the next 10 min. The whole cell lysate was used as a loading control for the 14-3-3ζ. The amount of the 14-3-3ζ was approximately the same in the whole cell lysate for the first 20 min. I-BOP appeared to stimulate the recruitment of the 14-3-3ζ to bind to TPα. Following 10 min of the stimulation, the 14-3-3ζ was slowly dissociated from the TPα. Similarly, the Raf-1 has some basal level of binding to the TPα before stimulation as shown in Fig. 3b. The binding also showed steady increase within the first 10 min of stimulation and began to decrease in the next 10 min. The total level of Raf-1 appeared to be constant during the stimulation.

Since the identification of 14-3-3 proteins as regulators of protein kinase C in the early 1990s, the 14-3-3 proteins have attracted attention for their role in controlling signal transduction process. Experiments were then performed to examine the signal transduction process induced by I-BOP.

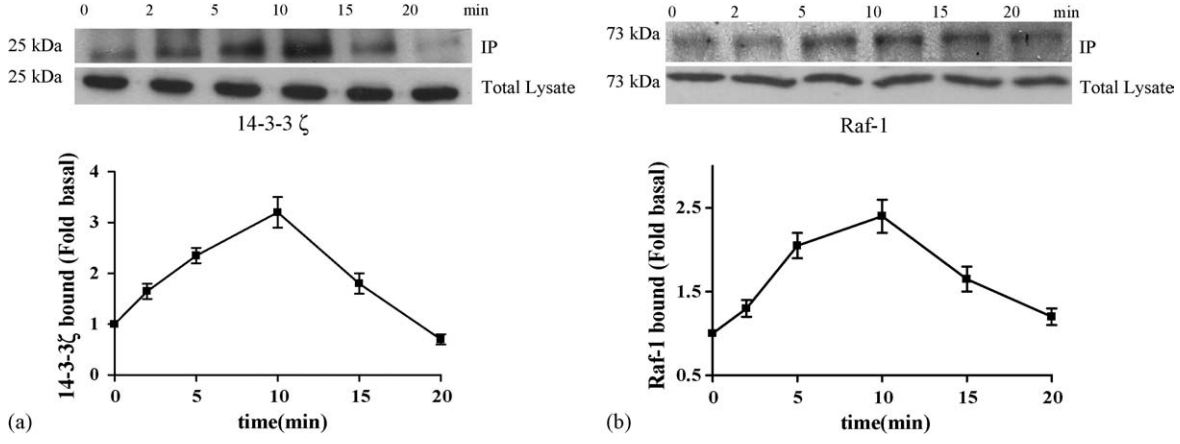

Fig. 3 – The association and dissociation process of the 14-3-3ζ (a) and the Raf-1 (b) with the TPα upon the agonist stimulation. After starvation for 16 h, the HEK293-TPα cells were stimulated with 50 nM I-BOP for 2, 5, 10, 15, 20 min. The cells then were quenched on ice and treated with lysis buffer at 4 °C. Twenty percent of the total protein from the lysate was directly analyzed by Western blotting to ensure an equal amount of protein (bottom panel in both a and b) was applied. Eighty percent of the total protein from the lysate was used to perform TP co-immunoprecipitation experiments as described in Section 2. The 14-3-3ζ and the Raf-1 were detected by their specific polyclonal antibodies (top panel in both a and b). The experiments were performed three times, and the band density of the 14-3-3ζ (a) and the Raf-1 (b) normalized to total lysates bands in terms of fold-increase over basal were plotted as a function of time. IP: immunoprecipitation; total lysate: the whole cell lysates used to perform the immunoprecipitation.

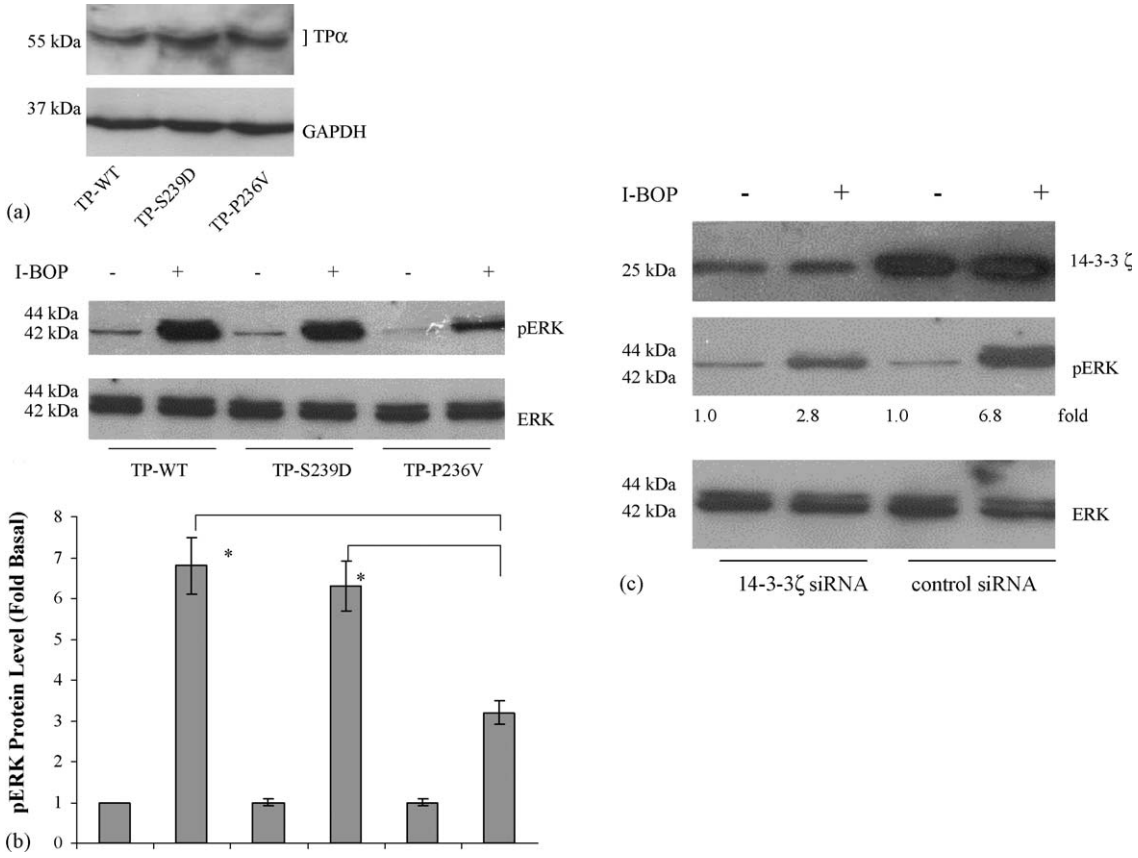

Fig. 4 – 14-3-3ζ is an important component in the agonist-induced ERK activation mediated by TPα. (a) Wild type and mutants of TPα expression in HEK293 cells. The constructs pcDNA3-TPα, pcDNA3-TPα-P236V, and pcDNA3-TPα-S239D were transiently transfected to HEK293 cells by lipofectamine reagent as mentioned in Section 2; following growth in DMEM media containing 10% FBS for 24 h. (b) Wild type and mutants of TPα-mediated agonist-induced ERK activation. After starvation in DMEM media without FBS for 16 h, the cells transiently transfected with wild type and mutants of TPα were stimulated with 50 nM I-BOP for 15 min. Protein lysates from transfected cells were separated by SDS-PAGE and analyzed by Western blotting using anti-pERK antibody. The same membrane was re-probed by ERK polyclonal antibody to ensure the equal amount of total protein was used. Densitometry of pERK was also performed (lower panel) and normalized to ERK. Data are presented as fold of basal ERK1/2 phosphorylation, in which the ERK1/2 phosphorylation in non-stimulated HEK293 cells transiently transfected with wild type of TPα was defined as 1.0. Values shown represent mean ± S.E. from three independent experiments. Asterisk indicates $P < 0.05$, compared between the two groups indicated in the figure by Student's t-test. (c) Blockade of 14-3-3ζ expression by 14-3-3ζ siRNA attenuated TP agonist-induced ERK activation in HEK293-TPα cells. After transfection with siRNA as described under Section 2, the cells were allowed to grow in DMEM media containing 10% FBS for 48 h, followed by starvation in DMEM media without FBS for 16 h, and then the cells were stimulated with 50 nM I-BOP for 15 min. The 14-3-3ζ (top panel), pERK (middle panel), and total ERK (bottom panel) were detected as described in (b). The pERK bands were scanned and the densities normalized to the ERK were presented by fold-increase over basal. 14-3-3ζ siRNA: transfection with 14-3-3ζ siRNA; control siRNA: transfection with non-specific siRNA provided by the vendor. Results are representative of three independent experiments.

P236V and S239D mutants and the wild type receptors were each transiently expressed in relatively equal amount in HEK293 cells (Fig. 4a). I-BOP was used to induce the activation of ERK. The results showed that I-BOP induced less phosphorylation of ERK in cells expressing P236V mutant receptor than in those expressing the wild type or the S239D mutant receptors (Fig. 4b). This is not surprising since the P236V mutant interacts poorly as shown above with the 14-3-3ζ, which is needed for the activation of ERK. This finding was further supported by the siRNA study in which the 14-3-3ζ siRNA was used to attenuate the expression of the 14-3-3ζ in HEK293-TPα cells. I-BOP-induced ERK phosphorylation was found to be decreased in the 14-3-3ζ siRNA transfected cells (Fig. 4c).

4. Discussion

In this study, we discovered the 14-3-3ζ as a novel TPα interacting protein by yeast-two-hybrid approach and showed that the 14-3-3ζ was involved in the TP agonist-induced activation of the ERK. In vitro GST pull-down and in vivo co-

immunoprecipitation studies confirmed that the 14-3-3ζ bound to the i3 domain of the TPα. Accordingly, the 14-3-3ζ should also interact with the TPβ since both forms have the common sequence in the i3 domain. It has been shown that both α and β forms of the TP can mediate transactivation of ERK induced by TP agonists [5–7]. Therefore, our observations can be extended to the TPβ system. However, further studies are needed to identify the exact role of the 14-3-3ζ in the TPβ-initiated signal transduction pathway.

The 14-3-3 proteins are a family of conserved proteins found in all eukaryotic cells [28]. They function by: (a) acting as adaptor proteins to facilitate protein–protein interactions [29], (b) modulating the subcellular localization or distribution of proteins [25,30], and (c) activation or inhibition of enzymes [31]. The 14-3-3 ligand binding is often dependent on the phosphorylation of serine or threonine residues in the motif of RSX(pS/pT)XP or RXXX(pS/pT)XP, which often is Akt/PKB's substrate consensus sequence [23,24]. Our site-directed mutagenesis studies indicate that the Pro-236 is directly involved in the interaction, but the phosphorylation of Ser-236, the only Ser residue in the TP-i3, is not essential in the binding. It has been shown that the 14-3-3 dimer binds to a single protein at two sites [32]. One site is a dominant site or ''gatekeeper'', which is phosphorylated in unstimulated cells. The secondary site is unable to promote a stable 14-3-3ζ interaction when the dominant site is absent or not phosphorylated. Actually, there are many phosphorylation sites on the C-tails of both TPα and TPβ which can be targeted by PKA, PKC [33], and Akt/PKB (our unpublished data). Although our results show no direct interaction between the 14-3-3ζ and the TP-Ct in vitro by GST pull-down experiment, we cannot exclude the possibility of their in vivo interaction. Therefore, a dominant binding site could be on the C-tails of the TPα/β.

TPs belong to a G-protein-coupled receptor (GPCR) superfamily. Activation of GPCRs is known to induce transactivation of epidermal growth factor receptor (EGFR) leading to activation of ERK [34]. TPs have been reported to mediate transactivation of ERK cascades in ECV304 bladder cancer cells [6] and in HEK293 cells stably transfected with TPs [5]. The cross-talk between the TPs and EGFR is thought to be via PKC-dependent Gi coupling and Src-dependent phosphorylation of the EGFR [6]. Upon activation of EGFR, Ras/Raf/MEK/ERK cascade is then activated. Our finding that the 14-3-3ζ binds to TP-i3 prompted us to propose that the cross-talk between the two receptors is through the adaptor protein 14-3-3ζ since the 14-3-3ζ is known to be a partner protein of Raf-1 [15], one of the downstream kinase of EGFR. Although we demonstrated that TP antibody immunoprecipitated both 14-3-3ζ and Raf-1, we are not certain if TP binds to Raf-1 directly or indirectly through 14-3-3ζ. Other GPCRs, such as α_2-adrenergic receptor also binds to the 14-3-3ζ at the i3 and the interaction is suppressed by a phosphorylated Raf peptide but not by its non-phosphorylated counterpart [22]. The 14-3-3ζ also facilitates the efficiency of the Ras-mediated signaling in *Drosophila* [35]. It has been postulated that the interaction of signaling molecules with 14-3-3 homo- or heterodimers serves as a scaffolding mechanism to facilitate interactions among molecular components of signaling cascades [36]. Our data show that both 14-3-3ζ and Raf-1 are components in the TP signaling complex, although our results cannot exclude the possibility that there is a direct binding of the Raf-1 to the TPs. Accumulating evidences support that the 14-3-3s serve as scaffold proteins in the cell signal transduction. Current study shows that the 14-3-3ζ and the raf-1 had a similar pattern of association and dissociation process with the TPα upon the agonist stimulation. Miggin and Kinsella [5,7] reported that U-46619-induced ERK activation was totally blocked by wortmannin, an inhibitor of PI 3-kinase, suggesting that PI-3 kinase/Akt pathway is involved in the ERK activation. These authors also demonstrated that PI-3 kinase was associated with the TPs, although they were not sure if the PI-3 kinase bound to TPs directly. Previously, PI-3 kinase was found to be an interaction protein of 14-3-3ζ and forms a complex with platelet membrane glycoprotein Ib-IX-V to promote the rapid translocation of these signaling proteins to the activated cytoskeleton [25]. It appears that the 14-3-3ζ links several of these components together to form a large protein complex and has important roles in the translocation of the signaling proteins to subcellular compartment.

Recently, Hekman et al. [16] proposed a model showing the roles of 14-3-3ζ and the Raf-1 in response to growth factor stimulation. In this model, the association of the Raf-1 with the plasma membrane represents an important step in the Raf-1 activation. Transition from the inactive to the active membrane-associated form of Raf-1 involves the Raf-1 dimerization, and the complex formation between the Raf-1 and the 14-3-3ζ. Taken the model reported by Hekman et al. [16] and our results together, we propose that upon TP's activation, Raf-1 is recruited to the plasma membrane and interacts with the TP through the 14-3-3ζ, which serves as a bridge to connect the TP and the Raf-1. Subsequently, the 14-3-

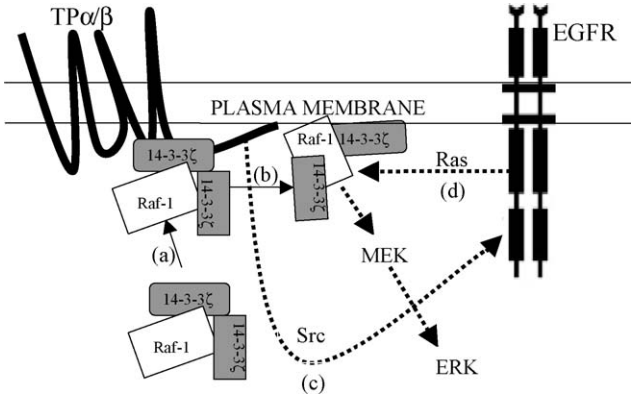

Fig. 5 – Proposed model of the role of 14-3-3ζ in the cross-talk between the TPα/β and the EGFR. Upon agonist stimulation, the complex of the 14-3-3ζ and the Raf-1 translocates from the cytosol to the plasma membrane and interacts with the TPα/β (a). Then, the complex of the14-3-3ζ and the Raf-1 is dissociated from the TPα/β and binds directly to the plasma membrane through an unknown mechanism (b). At the same time, TP activates EGFR through Src kinase (c). The activated EGFR will trigger ERK activation (d), in which the 14-3-3ζ facilitates Raf-1 activation through its translocation. Solid lines and arrows indicate protein complex translocation; dotted lines and arrows indicate direction of signal transduction pathways.

3ζ and the Raf-1 are dissociated from the TP following the transactivation the EGFR through Src. Finally, the Raf-1 is activated by Ras leading to the phosphorylation of the downstream ERK. A schematic model of these signal transduction processes is depicted in Fig. 5.

In conclusion, we identified 14-3-3ζ as a novel interaction protein with the TPs. The 14-3-3ζ may serve as a scaffold protein to construct TP/14-3-3ζ/Raf-1 protein complex and facilitate the translocation of signal proteins to certain subcellular compartment. Upon agonist stimulation, the 14-3-3ζ helps recruiting the Raf-1 from the cytosol to the plasma membrane and binds to the TPs. This protein complex is coupled to the MAPK pathway through the Raf-1, which is downstream of the EGFR. The 14-3-3 proteins are long time known as factors of anti-apoptosis [37]. Our results provide some evidence of their roles in the cell proliferation through MAPK pathway.

Acknowledgements

This study was supported in part by the Kentucky Lung Cancer Research Program and the NIH Grant HL-46296.

REFERENCES

[1] Hanasaki K, Nakano T, Arita H. Receptor-mediated mitogenic effect of thromboxane A_2 in vascular smooth muscle cells. Biochem Pharmacol 1990;40:2535–42.
[2] Narumiya S, Sugimoto Y, Ushikubi F. Prostanoid receptors: structures, properties, and functions. Physiol Rev 1999;79:1193–226.
[3] Shen RF, Tai HH. Thromboxanes: synthase and receptors. J Biomed Sci 1998;5:153–72.
[4] Hirata M, Hayashi Y, Ushikubi F, Yokota Y, Kageyama R, Nakanishi S, et al. Cloning and expression of cDNA for a human thromboxane A_2 receptor. Nature 1991;49:617–20.
[5] Miggin SM, Kinsella BT. Regulation of extracellular signal-regulated kinase cascades by alpha- and beta-isoforms of the human thromboxane A_2 receptor. Mol Pharmacol 2002;61:817–31.
[6] Gao Y, Tang S, Zhou S, Ware JA. The thromboxane A_2 receptor activates mitogen-activated protein kinase via protein kinase C-dependent Gi coupling and Src-dependent phosphorylation of the epidermal growth factor receptor. J Pharmacol Exp Ther 2001;296:426–33.
[7] Miggin SM, Kinsella BT. Thromboxane A_2 receptor mediated activation of the mitogen activated protein kinase cascades in human uterine smooth muscle cells. Biochim Biophys Acta 2001;1539:147–62.
[8] McGinty A, Foschi M, Chang YW, Han J, Dunn MJ, Sorokin A. Induction of prostaglandin endoperoxide synthase 2 by mitogen-activated protein kinase cascades. Biochem J 2000;352:419–24.
[9] Gallet C, Blaie S, Levy-Toledano S, Habib A. Epidermal-growth-factor receptor and metalloproteinases mediate thromboxane A_2-dependent extracellular-signal-regulated kinase activation. Biochem J 2003;371:733–42.
[10] Tzivion G, Shen YH, Zhu J. 14-3-3 proteins: bringing new definitions to scaffolding. Oncogene 2001;20:6331–8.
[11] Konishi Y, Lehtinen M, Donovan N, Bonni A. Cdc2 phosphorylation of BAD links the cell cycle to the cell death machinery. Mol Cell 2002;9:1005–16.
[12] Basu S, Totty NF, Irwin MS, Sudol M, Downward J. Akt phosphorylates the Yes-associated protein, YAP, to induce interaction with 14-3-3 and attenuation of p73-mediated apoptosis. Mol Cell 2003;11:11–23.
[13] Gohla A, Bokoch GM. 14-3-3 regulates actin dynamics by stabilizing phosphorylated cofilin. Curr Biol 2002;12:1704–10.
[14] Hermeking H. The 14-3-3 cancer connection. Nat Rev Cancer 2003;3:931–43.
[15] Freed E, Symons M, Macdonald SG, McCormick F, Ruggieri R. Binding of 14-3-3 proteins to the protein kinase Raf and effects on its activation. Science 1994;265:1713–6.
[16] Hekman M, Wiese S, Metz R, Albert S, Troppmair J, Nickel J, et al. Dynamic changes in C-Raf phosphorylation and 14-3-3 protein binding in response to growth factor stimulation: differential roles of 14-3-3 protein binding sites. J Biol Chem 2004;279:14074–86.
[17] Zhou H, Yan F, Tai HH. Phosphorylation and desensitization of the human thromboxane receptor-alpha by G protein-coupled receptor kinases. J Pharmacol Exp Ther 2001;298:1243–51.
[18] Ding Y, Tong M, Liu S, Moscow JA, Tai HH. NAD^+-linked 15-hydroxyprostaglandin dehydrogenase (15-PGDH) behaves as a tumor suppressor in lung cancer. Carcinogenesis 2005;26:65–72.
[19] Chiang N, Kan WM, Tai HH. Site directed mytagenesis of cysteinyl and serine residues of human thromboxane A_2 receptor in insect cells. Arch Biochem Biophys 1996; 334:9–17.
[20] Yamamoto S, Yan F, Zhou H, Tai HH. Agents that elevate cyclic AMP induce receptor phosphorylation primarily at serine 331 in HEK 293 cells overexpressing human thromboxane receptor alpha. Biochem Pharmacol 2002;64:375–83.
[21] Braman J, Papworth C, Greener A. Site-directed mutagenesis using double-stranded plasmid DNA templates. Methods Mol Biol 1996;57:31–44.
[22] Prezeau L, Richman JG, Edwards SW, Limbird LE. The zeta isoform of 14-3-3 proteins interacts with the third intracellular loop of different alpha2-adrenergic receptor subtypes. J Biol Chem 1999;274:13462–9.
[23] Yaffe MB, Rittinger K, Volinia S, Caron PR, Aitken A, Leffers H, et al. The structure basis for 14-3-3: phosohopeptide binding specificity. Cell 1997;91:961–71.
[24] Chen HK, Fernandez-Funez P, Acevedo SF, Lam YC, Kaytor MD, Fernandez MH, et al. Interaction of Akt-phosphorylated ataxin-1 with 14-3-3 mediates neurodegeneration in spinocerebellar ataxia type 1. Cell 2003;113:457–68.
[25] Munday AD, Berndt MC, Mitchell CA. Phosphoinositide 3-kinase forms a complex with platelet membrane glycoprotein Ib-IX-V complex and 14-3-3zeta. Blood 2000;96:577–84.
[26] Meller N, Liu YC, Collins TL, Bonnefoy-Berard N, Baier G, Isakov N, et al. Direct interaction between protein kinase C theta (PKC theta) and 14-3-3 tau in T cells: 14-3-3 overexpression results in inhibition of PKC theta translocation and function. Mol Cell Biol 1996;16: 5782–91.
[27] McPherson RA, Harding A, Roy S, Lane A, Hancock JF. Interactions of c-Raf-1 with phosphatidylserine and 14-3-3. Oncogene 1999;18:3862–9.
[28] Muslin AJ, Xing H. 14-3-3 proteins: regulation of subcellular localization by molecular interference. Cell Signal 2002;12:703–9.
[29] Zhao X, Gan L, Pan H, Kan D, Majeski M, Adam SA, et al. Multiple elements regulate nuclear/cytoplasmic shuttling of FOXO1: characterization of phosphorylation- and 14-3-3-

dependent and -independent mechanisms. Biochem J 2004;378(Pt 3):839–49.
[30] Konishi Y, Lehtinen M, Donovan N, Bonni A. Cdc2 phosphorylation of BAD links the cell cycle to the cell death machinery. Mol Cell 2002;9:1005–16.
[31] Ganguly S, Weller JL, Ho A, Chemineau P, Malpaux B, Klein DC. Melatonin synthesis: 14-3-3-dependent activation and inhibition of arylalkylamine *N*-acetyltransferase mediated by phosphoserine-205. Proc Natl Acad Sci USA 2005;102:1222–7.
[32] Michael B, Yaffe A. How do 14-3-3 proteins work? Gatekeeper phosphorylation and the molecular anvil hypothesis. FEBS Lett 2002;513:53–7.
[33] Habib A, FitzGerald GA, Maclouf J. Phosphorylation of the thromboxane receptor alpha, the predominant isoform expressed in human platelets. J Biol Chem 1999;274: 2645–51.
[34] Daub H, Weiss FU, Wallace C, Ullrich A. A role of transactivation of the EGF receptor in signaling by G-protein-coupled receptors. Nature 1996;379: 557–60.
[35] Chang HC, Rubin GM. 14-3-3 epsilon positively regulates Ras-mediated signaling in *Drosophila*. Genes Dev 1997;11:1132–9.
[36] Fanger GR, Widmann C, Porter AC, Sather S, Johnson GL, Vaillancourt RR. 14-3-3 proteins interact with specific MEK kinases. J Biol Chem 1998;273:3476–83.
[37] Xing H, Zhang S, Weinheimer C, Kovacs A, Muslin AJ. 14-3-3 proteins block apoptosis and differentially regulate MAPK cascades. EMBO J 2000;19:349–58.