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Thromboxane A_2-induced signal transduction is negatively regulated by KIAA1005 that directly interacts with thromboxane A_2 receptor

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ABSTRACT

Thromboxane A_2 (TXA_2), a potent inducer of platelet aggregation and smooth muscle contraction, exerts its action through TXA_2 receptor (TP). There are two alternative splicing variants of TP, TPα and TPβ. To clarify the signal transduction of TP pathway, we searched for putative TP binding proteins using a yeast two-hybrid system with the C-terminal region of TPα or TPβ as bait. We found KIAA1005 as a novel interacting protein of the TPα and TPβ C-terminal region (TP interacting protein, TPIP). KIAA1005/TPIP was co-immunoprecipitated with TPα or TPβ in HEK293 cells expressing myc-KIAA1005/TPIP and FLAG-TP isoforms. Expression analysis showed a ubiquitous expression pattern of KIAA1005/TPIP mRNA, including prominent expression in the thymus. Furthermore, TP-mediated phosphoinositide hydrolysis, phosphorylation of extracellular signal-regulated kinase (ERK) 1/2 and interleukin-6 production were reduced by the expression of KIAA1005/TPIP. The expression of KIAA1005/TPIP decreased cell-surface TPα and TPβ levels. Thus, we show for the first time that KIAA1005/TPIP is a novel TP interacting protein that regulates TP-mediated signal transduction negatively.

1. Introduction

Thromboxane A_2 (TXA_2) plays an important role in platelet aggregation/thrombus formation, constriction and mitogenesis of vascular and bronchial smooth muscle cells, and its action is mediated via the TXA_2 receptor (TP) [1–3]. TP is a member of the G protein-coupled receptor (GPCR) family and is shown to be coupled with $G_{q/11}$ [4–6]. Furthermore, TP has been reported to communicate with other G proteins, including G_{12}, G_{13}, G_i, G_s and G_h, thus activating multiple signaling pathways [1,7–9]. Human TP is expressed from a single gene [10] that has two alternatively spliced isoforms, TPα and TPβ [11,12]. TPα cDNA encoding a 343-amino acid protein was cloned from human placental cells [11], while TPβ cDNA encoding a 407-amino acid protein was cloned from human endothelial cells [12]. While TPα and TPβ share the first 328 amino acids, these isoforms have different C termini. The predicted ligand-binding domains are the same for both receptor isoforms, which show the same affinity for ligands [13].

It is generally believed that the third intracellular loop and C-terminal domain of GPCR are important for coupling with heterotrimeric G proteins [14,15]. Thus, the difference in the C-terminal domain of the two TP variants is likely to show a difference in G protein coupling or receptor desensitization [16]. In fact, TP agonists cause the elevation of cAMP in CHO cells expressing TPα but not TPβ, while they cause phosphoinositide hydrolysis to a similar extent in cells expressing TPα and TPβ. On the other hand, it has been shown that stimulation of TP causes homologous desensitization in 1321N1 human astrocytoma cells [17]. Habib et al. [18] reported that U46619, a TP agonist, induces phosphorylation and homologous desensitization of both TP isoforms. In contrast, there are several reports showing that TPα and TPβ are desensitized differently [19,20]. These observations suggest that the C terminus of TPα and TPβ is important for inducing G protein coupling and desensitization.

It was recently reported that numerous proteins interact with the C terminus of GPCR, and these proteins regulate GPCR signaling. The most well known protein that interacts with the C terminus of GPCR is β-arrestin, which binds to GPCR kinase (GRK)-phosphorylated receptors and can terminate receptor-G protein coupling [21], and internalize the receptor into clathrin-coated vesicles [22]. Small GTPase ARF4, a member of the ARF family of protein sorting regulators, interacts with the C-terminus of rhodopsin. ARF4

Abbreviations: CORS, cerebello-oculo-renal syndrome; DMEM, Dulbecco's modified Eagle's medium; ERK, extracellular signal-regulated kinase; FCS, fetal calf serum; GA3PDH, glyceraldehyde-3-phosphate dehydrogenase; GPCR, G protein-coupled receptor; HRP, horseradish peroxide; MKS, Meckel syndrome; PIP_2, phosphatidylinositol 4,5-bisphosphate; PKC, protein kinase C; PTHR, parathyroid hormone receptor; PVDF, polyvinylidene difluoride; TCA, trichloroacetic acid; TP, thromboxane A_2 receptor; TXA_2, thromboxane A_2.

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was reported to be essential for rhodopsin sorting from post-Golgi to the rod outer segments [23]. In other reports, t-complex testis expressed-1 (Tctex-1), 4.1G and 14-3-3 proteins were found to be associated with the C-terminus of the parathyroid hormone receptor (PTHR) [24–26]. Tctex-1, one of the light chains of cytoplasmic dynein, was involved in agonist-induced internalization of PTHR [26]. 4.1G, a cytoskeletal protein, facilitated the cell-surface localization of PTHR and augmented PTHR-mediated signal transduction [24]. 14-3-3 protein, a molecular scaffolding protein, caused localization of the receptor to the nucleus from the plasma membrane [25]. Previously, we identified proteasome subunit α7 and proteasome activator PA28γ as TPβ associated proteins that regulate the receptor transport to the plasma membrane [13].

In the present study, we identified KIAA1005 as a protein associated with the C terminus of TPα and TPβ. Furthermore, we examined the regulatory mechanism of the novel TP interacting protein KIAA1005 in TP-mediated signal transduction.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium was obtained from Nissui Pharmaceutical (Tokyo, Japan). U46619 and SQ29548 were purchased from Cayman Chemical (Ann Arbor, MI, USA). [^{3}H]SQ29548 and myo-[^{3}H]inositol were from PerkinElmer Life Sciences (Boston, MA, USA). The expression vector pFLAG-CMV4 and anti-FLAG antibody were from Sigma (St. Louis, MO, USA). The expression vector pcDNA3.1+, ProQuest Two-Hybrid System, human adult brain cDNA library, and Lipofectamine 2000 were from Invitrogen (Merelbeke, Belgium). The Human Total RNA Master Panel II was from Clontech (Palo Alto, CA, USA). The HRP-conjugated anti-mouse IgG, and ECL reagent were from Amersham Biosciences (Tokyo, Japan). PrimeSTAR^{TM} HS DNA Polymerase was from TAKARA BIO (Shiga, Japan). Quik Change Site-Directed Mutagenesis Kit was from Stratagene (Tokyo, Japan). HRP-conjugated anti-rabbit IgG was from Cell Signaling Technology (Beverly, MA, USA) and Mouse TrueBlot HRP anti-mouse IgG was from eBioscience (San Diego, CA, USA).

2.2. Cell culture and transfection

CHO cells and HEK293 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 50 units/ml penicillin, and 50 mg/ml streptomycin in a 5% CO_2 atmosphere at 37 °C [27]. Human astrocytoma cells (1321N1) were grown in DMEM supplemented with 5% fetal calf serum, 50 units/ml penicillin, and 50 mg/ml streptomycin in a 5% CO_2 atmosphere at 37 °C [28]. Transfection was performed with Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions.

2.3. Plasmid construction

KIAA1005/TPIP was obtained by RT-PCR from human brain cDNA liberally, using primers 5′-GCGAGGAATTCATGTCTGGTCCAACTGATGAGAC-3′ and 5′-GCTACTGAATT CTCAAGCCTCCAAGTCATCTCTG-3′, including two EcoRI sites. The amplified KIAA1005/TPIP fragment was digested with EcoRI, and was inserted into myc-pcDNA3.1+. The cDNAs encoding wild-type TPα (HPL/pBluescript) was a generous gift from Dr. S. Narumiya (Kyoto University, Kyoto), and TPα/pcDNA3.1(+) was constructed by inserting their cDNAs digested with BamHI/HincII into pcDNA3.1(+) digested with BamHI/EcoRV. The plasmid encoding wild-type TPβ was constructed by inserting two fragments into pcDNA3.1(+). The first fragment was obtained by digestion of HPL/pBluescript at the EcoRI/BsiHKAI site. The second fragment was obtained by RT-PCR using RNA from 1321N1 human astrocytoma cells with a sense primer (5′-AAAGTCGACAAGAGCCGTGCTCAGGC GTCTCAGCC-3′) and an antisense primer (5′-TTGCGG CCGCTCAATCCTTTCTGGACAGAGCCTTCCC-3′), and the fragment was digested with BsiHKAI/NotI. The resulting cDNA fragments were cloned into pcDNA3.1(+) digested with EcoRI/NotI together. To construct the FLAG-tagged receptors, WT-TPα/pcDNA3.1+ and WT-TPβ/pcDNA3.1+ were digested with ApaI and XbaI. The DNA fragment of TP and annealed oligonucleotides (5′-AGCTTTGGCCCAACGGCAGTTCCCTGGGCC-3′ and 5′-CCAGGGAACTGCCGTTGGGCCAA-3′) were ligated. Then, the ligated DNAs were inserted in-frame at the HindIII and XbaI site of pFLAG-CMV4. All PCR was performed using pfu turbo DNA polymerase (Stratagene) and the DNA sequence was confirmed using an ABI310 (Applied Biosystems).

2.4. Yeast two-hybrid screening

Yeast two-hybrid screening was carried out using the ProQuest Two-Hybrid System according to the manufacturer's instructions. Briefly, yeast cells (MaV203) containing bait plasmid TPαC-tail/pDBLeu or TPβC-tail/pDBLeu were transformed with a human adult brain cDNA library, using the lithium acetate method. Positive clones were screened on plates lacking leucine, tryptophan and histidine. Growth of the resulting colonies was analyzed on plates lacking uracil, and using the β-galactosidase assay. Plasmids recovered from positive colonies were introduced into *Escherichia coli* HB101, which were plated in the presence of ampicillin. Then, the plasmid DNA isolated from ampicillin-resistant colonies was identified by sequencing.

2.5. Immunoprecipitation

HEK293 cells transfected with myc-KIAA1005/TPIP and FLAG-TPα or FLAG-TPβ were incubated for 1 h at 4 °C in lysis buffer (150 mM NaCl, 0.1% NP40, 1 mM PMSF, 0.01% aprotinin, 20 μg/ml leupeptin, 20 mM Tris–HCl, pH 7.4). Lysates were centrifuged at 17,400 × *g* for 10 min at 4 °C, and supernatants were incubated for 4 h at 4 °C with anti-FLAG M2-agarose. Following incubation, the agarose beads were precipitated by centrifugation at 700 × *g* for 2 min and washed three times with lysis buffer and once with TBS.

2.6. Pull-down assay

For expression of GST fusion proteins, the recombinant TPαC-tail/pGEX-5X-2 and TPβC-tail/pGEX-5X-2 were introduced into *E. coli* BL21, and fusion proteins were induced by isopropyl β-D-thiogalactoside. Then, the proteins of transformed *E. coli* were collected in buffer A [20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM DTT, Protease Inhibitor Cocktail (Roche), 10 mM EDTA, 2 mM PMSF]. For preparation of myc-KIAA1005/TPIP, HEK293 cells transfected with myc-KIAA1005/TPIP were lysed by the incubation for 1 h at 4 °C in buffer B (20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM DTT, 0.1% NP40). GST fusion protein (15 μg) and HEK293 cell lysate (100 μg) expressing myc tag-fused KIAA1005/TPIP were incubated in buffer B overnight at 4 °C. Then, the protein mixtures were incubated with glutathione-sepharose beads for 1 h at room temperature. The beads were washed three times with buffer B, and washed twice with buffer C (buffer B without NP40). The protein-bound beads were precipitated by centrifugation, and suspended in SDS-PAGE sample buffer. The samples were subjected to 11% SDS-PAGE.

2.7. Western blot analysis

To analyze extracellular signal-regulated kinase1/2 (ERK1/2) activity, TPα-CHO and TPβ-CHO cells were seeded onto 12-well plates at a density of 1.0 × 10^5 cells/well. Twenty-four hour after