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Low expression of cell-surface thromboxane A_2 receptor β-isoform through the negative regulation of its membrane traffic by proteasomes

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Abstract

Human thromboxane A_2 receptor (TP) consists of two alternatively spliced isoforms, TPα and TPβ, which differ in their cytoplasmic tails. To examine the functional difference between TPα and TPβ, we searched proteins bound to C termini of TP isoforms by a yeast two-hybrid system, and found that proteasome subunit α7 and proteasome activator PA28γ interacted potently with the C terminus of TPβ. The binding of TPβ with α7 and PA28γ was confirmed by co-immunoprecipitation and pull-down assays. MG-132 and lactacystin, proteasome inhibitors, increased cell-surface expression of TPβ, but not TPα. Scatchard analysis of [^{3}H]SQ29548 binding revealed that the B_{max} was higher in transiently TPα-expressing cells than TPα-expressing cells. In addition, TP-mediated phosphoinositide hydrolysis was clearly observed in TPα-, but not TPβ-expressing cells. These results suggest that TPβ binds to α7 and PA28γ, and the cell-surface expression of TPβ is lower than that of TPα through the negative regulation of its membrane traffic by proteasomes.
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1. Introduction

Thromboxane A_2 (TXA_2) plays an important role in constriction of vascular and bronchiolar smooth muscle cells, mitogenesis of vascular smooth muscle cells, and platelet aggregation through stimulation of TXA_2 receptor (TP)

Abbreviations: CFTR, cystic fibrosis transmembrane conductance regulator; ER, endoplasmic reticulum; ERAD, ER-associated degradation; GPCR, G protein-coupled receptor; GST, glutathione S-transferase; HRP, horseradish peroxide; PVDF, polyvinylidene difluoride; TCA, trichloroacetic acid; TP, thromboxane A_2 receptor; TXA_2, thromboxane A_2

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[1]. TP is a member of the G protein-coupled receptor (GPCR) family and is known to be coupled with $G_{q/11}$ [2–4]. Furthermore, TP has been reported to communicate with other G proteins, such as G_{12}, G_{13}, G_i and G_h [5–7], thus evoking the activation of multiple signaling pathways. However, the switching mechanism of TP in communication with these different G proteins remains to be elucidated. Human TP has been found as a single gene that has two alternatively spliced isoforms, TPα and TPβ. TPα cDNA encoding a protein of 343 amino acids was cloned from human placental cells [8], and TPβ cDNA encoding 407 amino acids was cloned from human endothelial cells [9]. While TPα and TPβ share the first 328 amino acids, these isoforms are different in their C termini. The predicted ligand-binding domains are the same for both receptor isoforms [10]. In fact, it has been reported that there is no difference in the affinity for ligands between the two isoforms [4,11,12].

There are many human cells and tissues which express both TPα and TPβ [13], although TPα was originally found in placental cells [8] and TPβ in endothelial cells [9]. It is generally believed that the third intracellular loop and C-terminal domain of GPCR are important for coupling with heterotrimeric G protein, and for receptor desensitization through phosphorylation. 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. For instance, rat PACAP receptor splice variants with insertions at the third intracellular loop show altered patterns of adenylyl cyclase and phospholipase C stimulation, through changes in coupling with G proteins [14]. Alternative splicing isoforms of prostanoid EP3 receptor, which differ only at their C-terminal tails, couple to different G proteins to activate different second messenger systems [15]. In the case of TP, Hirata et al. [4] have demonstrated that TP agonists cause 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. However, the molecular mechanism in coupling of alternative splicing variants of TP to the different G proteins remains to be established. On the other hand, it has been shown that stimulation of TP causes a homologous desensitization in 1321N1 human astrocytoma cells [16]. Habib et al. [17] have demonstrated that U46619, a TP agonist, induces phosphorylation and homologous desensitization of both TP isoforms to a similar extent, using isoform-specific antibodies. In contrast, there are several reports showing that TPα and TPβ are desensitized differently. For example, the number of TXA_2-binding sites is decreased in fibroblasts expressing TPα, by prolonged stimulation with a TP agonist, but those in cells expressing TPβ are markedly increased [12]. In addition, it has been shown that TPβ, but not TPα, undergoes both agonist-dependent and agonist-independent internalization [18,19]. These observations clearly indicate that there is a difference in the function and/or regulatory mechanisms of TPα and TPβ.

In the present study, we searched the proteins associated with the C terminus of TPα and/or TPβ in a yeast two-hybrid screening, and found that the C terminus of TPβ could directly bind to proteasome subunit α7 and proteasome activator PA28γ. In addition, we found that the cell surface expression of TPβ was negatively regulated through membrane traffic by proteasomes.

2. Materials and methods

2.1. Cell culture and transfection

CHO cells and HEK293 cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum in a humidified incubator with a 5% CO_2 atmosphere at 37 °C. Transfection was performed with Polyfect Transfection Reagent or Lipofectamine 2000, according to the manufacturer's recommendation.

2.2. Plasmid construction

WT-TPα/pcDNA3.1+ was generated by inserting the TPα fragment from HPL/pBluescript, kindly provided by Dr. S. Narumiya (Kyoto University, Japan), digested with *Bam*HI and *Hin*cII into pcDNA3.1+ digested with *Bam*HI and *Eco*RV. WT-TPβ/pcDNA3.1+ was obtained by inserting TPβC-tail cDNA obtained from RT-PCR of 1321N1 human astrocytoma cell lines. Primers 5′-AAAGTCGACAAGAGCCGTGCTCAGGCGTCTCCAGCC-3′ (TP1) and 5′-TTGCGGCCGCTCAATCCTTTCTGGACAGAGCCTTCCC-3′ were used for generation of TPβC-tail, including *Sal*I and *Not*I sites. Amplified TPβC-tail fragment was digested with *Bsi*HKAI and *Not*I, and inserted, together with a fragment from HPL/pBluescript digested with *Eco*RI and *Bsi*HKAI, into pcDNA3.1+ digested with *Eco*RI and *Not*I.

For construction of FLAG-tagged receptors, WT-TPα/pcDNA3.1+ and WT-TPβ/pcDNA3.1+ were digested with *Apa*I and *Xba*I. The DNA fragment of TP and annealed oligonucleotides (5′-AGCTTTGGCCCAACGGC-

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