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MOUSE THROMBOXANE A_2 RECEPTOR: cDNA CLONING, EXPRESSION AND NORTHERN BLOT ANALYSIS*

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A cDNA clone for the mouse thromboxane A_2 receptor was isolated from mouse lung cDNA library. The cDNA has a 1,023 base pair open reading frame which encodes a protein of 341 amino acid residues. STA_2 and U-46619 induced inward current in *Xenopus laevis* oocytes injected with the transcript of the clone. Specific binding of [^{3}H]S-145 was found in membranes of COS-1 cells transfected with the cDNA (Kd=3.3nM) and was displaced with unlabeled prostaglandins and thromboxane analogues in the order of S-145 > STA_2 > U-46619 > PGD_2 > $PGF_{2\alpha}$ = PGE_2. Northern blot analysis demonstrated that thromboxane A_2 receptor mRNA is expressed abundantly in thymus, spleen and lung.

Thromboxane A_2 (TXA_2) is quite an unstable yet highly potent arachidonate metabolite (1). It is a potent stimulator of platelets and a potent bronchial and vascular smooth muscle constrictor (1,2). From these bioactivities, TXA_2 has been implicated in thromboembolic diseases and bronchial asthma (3) and the roles of TXA_2 in these pathophysiological processes have been examined extensively. Quantitative analyses of TX synthase in human tissues demonstrated that macrophages/monocytes contain as much amount of the synthase as platelets (4). Immunohistochemical studies show that TX synthase localizes abundantly in cells of those tissues as dendritic cells of spleen and thymus, bronchial epithelium, crypt epithelium of tonsil, liver Kupffer cells and Langerhans cells in skin (5). However, it is not known on which type of cells acts TXA_2 produced by these cells. TXA_2 acts on a specific receptor on cell surface to exert its action. We have recently cloned a cDNA for the human TXA_2 receptor (6). Tissue

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Abbreviations. TXA_2, thromboxane A_2; TX, thromboxane; PCR, polymerase chain reaction; PG, prostaglandin; PKC, protein kinase C.

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distribution analysis and cell type identification using this clone was unsuccessful due to the lack of cross-hybridization to mRNA of other species. We, therefore, have cloned a cDNA for the mouse TXA_2 receptor by PCR and hybridization screening. Using this clone, we examined its mRNA distribution in various mouse tissues. We also present here molecular structure of the mouse TXA_2 receptor in comparison with those of the human TXA_2 and the mouse PGE_2 receptors. This study will help to reveal novel pathophysiological roles of TXA_2 and contribute to identify structural requirements of the eicosanoid receptors.

MATERIALS AND METHODS

Amplification of a Mouse cDNA Fragment Homologous to the Human TXA_2 Receptor by PCR. Total RNA was prepared from 4-5 week old DDY-strain mouse lung by acid-guanidium-thiocyanate-phenol-chloroform method (7). First strand cDNA was synthesized from the RNA using random primer (Toyobo, Osaka, Japan) and Molony Murine Leukemia Virus reverse transcriptase (BRL). PCR primers, 5'-CTGGGATCCGCCATGGCCTCAGAGCGCTAC-3' and 5'-ACGGAATTCAAGACCAGAAGGGGCAGCCA-ACA-3', were designed based on the cDNA sequence corresponding to the putative IIIrd and VIth transmembrane domains of the human TXA_2 receptor, respectively. Amplification was performed on a Zymoreactor (Atto, Tokyo, Japan) with the first strand cDNA as a template. Reaction was carried out with 30 cycles of 1 min of denaturation at 95°C, 0.5 min of annealing at 60°C, and 1.5 min of extension at 72°C. A single 418 base pair cDNA fragment was amplified, and subcloned into pBluescript SK(+) (Stratagene). The DNA insert in an isolated clone was named LT3, radiolabeled by random priming method and used in plaque hybridization as a probe.

Molecular Cloning by Plaque Hybridization. Mouse lung cDNA was prepared by an oligo(dT) priming method using a cDNA synthesis kit (Amersham), size-selected (>1 kilobase) and inserted into the EcoRI site of λ-ZAPII vector DNA with EcoRI adaptors (New England Biolabs). Clones (4.8×10^5) derived from the cDNA library were transferred to nylon membranes (Hybond-N plus, Amersham) and screened by hybridization to LT3. Hybridization was carried out at 68°C in 6xSSC containing 5xDenhardt's solution, 0.5% SDS, 200 μg/ml heat-denatured salmon sperm DNA and the radiolabelled probe (2×10^6 cpm/ml) for 12 h. Filters were washed twice at 68°C in 2xSSC containing 1% SDS for 30 min. Three positive clones were picked up and converted to plasmids via *in vivo* excision method and sequenced. One of the clones was revealed to be a full length clone (ML36).

Expression in Xenopus Oocytes. HindIII-linearized ML36 was transcribed *in vitro* by T7 RNA polymerase in the presence of a cap analogue, 7-methyl GpppG (New England Biolabs) as described (8). The transcript was microinjected into isolated oocytes and incubated at 19°C in Barth's saline for 2 days. After incubation, the whole cell voltage of a oocyte was clamped at -60 mV and whole cell current was recorded in response to various PGs and TXA_2 agonists applied to the bath at 1 μM .

Expression in COS-1 Cells and Ligand Binding Assay. The EcoRI insert of ML36 was subcloned into pcDNAI (Invitrogen), and the plasmid was transfected into COS-1 cells by modified DEAE-dextran method (9). The transfected cells were harvested and homogenized. The 100,000 x g pellet of this homogenate was suspended and used as a membrane fraction. [^{3}H]S-145 binding in the membrane was determined as described previously (10). Displacement experiment was carried out with 10 nM [^{3}H]S-145 in the presence of various concentrations of compounds.

Northern Blot Analysis. Poly $(A)^+$ RNA was purified using Oligotex dT30 (Takara Shuzo, Kyoto, Japan) from total RNA of a mouse organ. Five μg of poly $(A)^+$ RNA from each tissue was separated by electrophoresis on a 1.2% agarose gel, and transferred

onto a nylon membrane (Hybond-N, Amersham). An antisense RNA probe was prepared with T3 RNA polymerase. Hybridization was carried out at 70°C in 5xSSC, 50% formamide, 5xDenhardt's solution, 0.2% SDS, 250 μg/ml heat-denatured salmon sperm DNA, 200 μg/ml yeast transfer RNA and 5 ng/ml of the RNA probe. The filter was washed in 0.1xSSC and 0.1% SDS twice at 70°C for 20 min. RNase treatment was performed as described (11,12).

RESULTS AND DISCUSSION

Fig. 1 shows nucleotide and deduced amino acid sequences of ML36. ML36 contained the 1.7 kilobase insert which had an open reading frame of 1,023 base pairs. The deduced amino acid sequence consists of 341 amino acids with a calculated molecular mass of 37,114. The hydrophobicity profile determined by Kyte and Doolittle method (13) indicated that it has seven hydrophobic segments like the human TXA_2 receptor, suggesting that it is also a G-protein-coupled rhodopsin-type receptor. Potential glycosylation sites (14) exist at Asn 4 and Asn16 in the amino terminal which are identical to those in the human TXA_2 receptor.

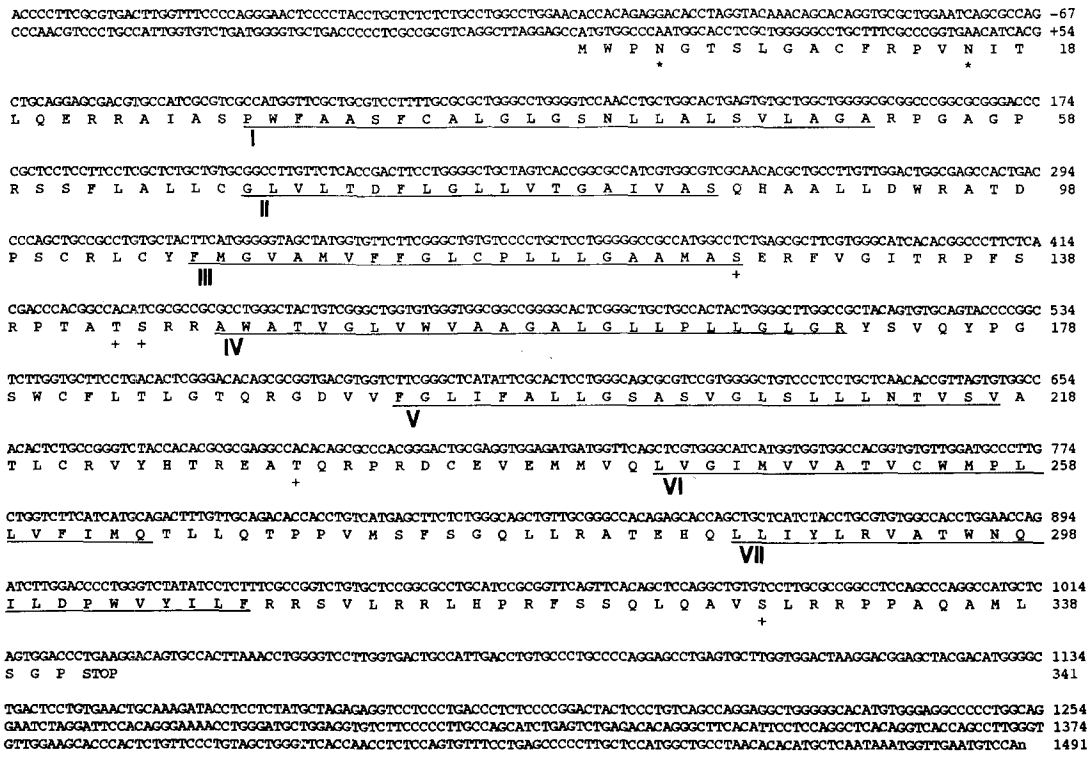

Fig.1. Nucleotide and deduced amino acid sequences of the mouse TXA_2 receptor cDNA. The nucleotide sequence is numbered in accordance with the first methionine of the longest open reading frame. The deduced amino acid sequence is shown beneath the nucleotide sequence in one letter code. Positions of the seven transmembrane domains (I-VII) are indicated by solid bars. *, potential N-glycosylation sites in the extracellular region. +, potential PKC-dependent phosphorylation sites in the intracellular regions.

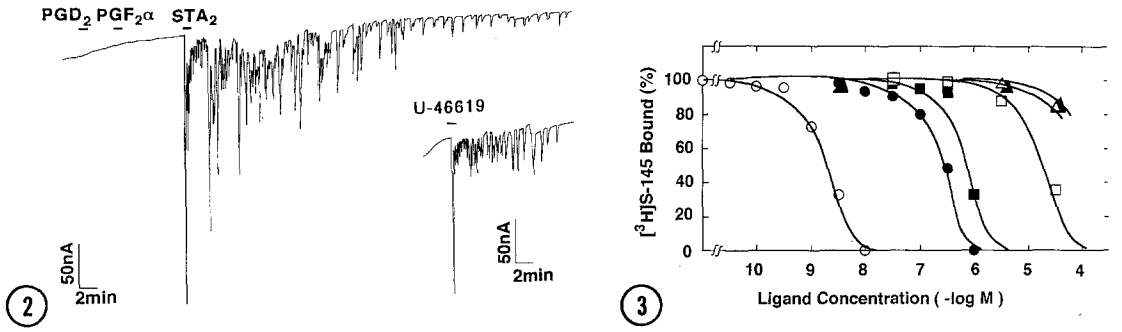

Fig.2. Current traces from Xenopus oocytes injected in vitro transcribed mouse TXA_2 receptor mRNA. Downward deflection indicates inward current. A bar above the traces shows duration of application of each drug at 1 μM. Two independent traces are shown.

Fig.3. Displacement of the specific binding of [^{3}H]S-145 in membranes of COS-1 cells transfected with the mouse TXA_2 receptor cDNA. Unlabeled TXA2 analogues and PGs are indicated as follows. S-145(○), STA2(●), U-46619(■), PGD_2(□), PGE_2(△), $PGF_{2\alpha}$(▲).

The TXA_2 receptor, when stimulated, evokes phosphatidylinositol turnover and mobilizes Ca^{2+} via inositol trisphosphate (15). In Xenopus oocyte the rise in intracellular Ca^{2+} is detected as an inward current induced by opening of Ca^{2+}-activated Cl^- channel (8). Fig. 2 shows electrophsiological responses of Xenopus oocytes injected the *in vitro* transcribed mRNA. The ocytes exhibited an inward current in response to TXA_2 agonists, STA_2 and U-46619. No response was evoked by other prostaglandins such as PGD_2, $PGF_{2\alpha}$, PGE_2 and iloprost (Fig. 2 and data not shown). These results demonstrate that ML36 encodes a functional TXA_2 receptor.

Membranes of the COS-1 cells transfected the cDNA showed specific binding to [^{3}H]S-145, a radioligand of the TXA_2 receptor. The Scatchard plot of the binding showed Kd and Bmax of 3.3 nM and 1.6 pmol/mg protein, respectively. Displacement of this binding is shown in Fig. 3. The binding of [^{3}H]S-145 was inhibited by unlabeled PGs and TX analogues in the order of S-145 > STA_2 > U-46619 > PGD_2 > $PGF_{2\alpha}$ = PGE_2. These binding characteristics were in good agreement with those observed in the receptor in other cells and the cloned human TXA_2 receptor (6,10,16).

Fig. 4 shows comparison of deduced amino acid sequences of the mouse TXA_2, the human TXA_2 (6) and the mouse PGE_2 receptors (17). The sequence of the mouse TXA_2 receptor is 76 % identical to that of the human TXA_2 receptor and the identity is 85% within the hydrophobic segments. The identity is only 33% between the mouse TXA_2 and the mouse PGE_2 receptors, and 42% within the hydrophobic segments of the two. The 7th putative transmembrane domain is the most homologous region in the hydrophobic segments of these three receptors. Potential phosphorylation sites by PKC (18) are indicated in outlined characters. There are five, four and three potential phosphorylation sites in the cytoplasmic structures of the mouse TXA_2, the human TXA_2 and the mouse PGE_2 receptors, respectively. Ser 127 and Ser 144 present in the 2nd

Fig.4. Comparison of the amino acid sequences among the mouse TXA_2 receptor, human TXA_2 receptor and mouse PGE_2 receptor. The amino acid sequences of the mouse TXA_2 receptor (MouseTX, upper), human TXA_2 receptor (HumanTX, middle) and mouse PGE_2 receptor (MouseEP, lower) are aligned to achieve the maximal homology using a computer program. Identical amino acids are boxed. Hyphens show deletions of amino acids when compared with other sequences. Outlined letters indicate potential PKC-dependent phosphorylation sites according to the consensus sequences. Positions of putative transmembrane domains were indicated as I-VII.

intracellular loop of the mouse TXA_2 receptor are conserved in the human TXA_2 receptors. These conserved sites may be involved in PKC-mediated heterologous desensitization of the TXA_2 receptor (19). There are several other serine and threonine residues common to the two TXA_2 receptors in the 1st, 2nd intracellular loops and the carboxyl terminal. They may be involved in agonist-induced homologous desensitization (19).

Fig. 5 shows the northern blot analysis of various mouse organs. A major band is observed at 2 kilobase (left arrow) with a minor band at 3 kilobase. Two bands were also observed in the northern blot analysis of the human TXA_2 receptor (6). Thymus is the organ richest in the TXA_2 receptor mRNA expression, followed by spleen and lung. Higher amount of expression is also observed in kidney, uterus, brain and heart, and little but significant expression is seen in liver, stomach, ileum and testis. High level expression in thymus and spleen strongly suggests that the TXA_2 receptor is involved in and modulates immune responses. Dendritic cells in thymus and spleen have as high TX synthase activity as platelets (4,5). However, its relevance to immunological modulation has not been fully elucidated (20). Only actions of TXA_2 on peripheral leukocytes are studied (21-24) and there has been no study examining roles of TXA_2 in cell to cell interaction in thymus and spleen. The present study suggests that TXA_2 has

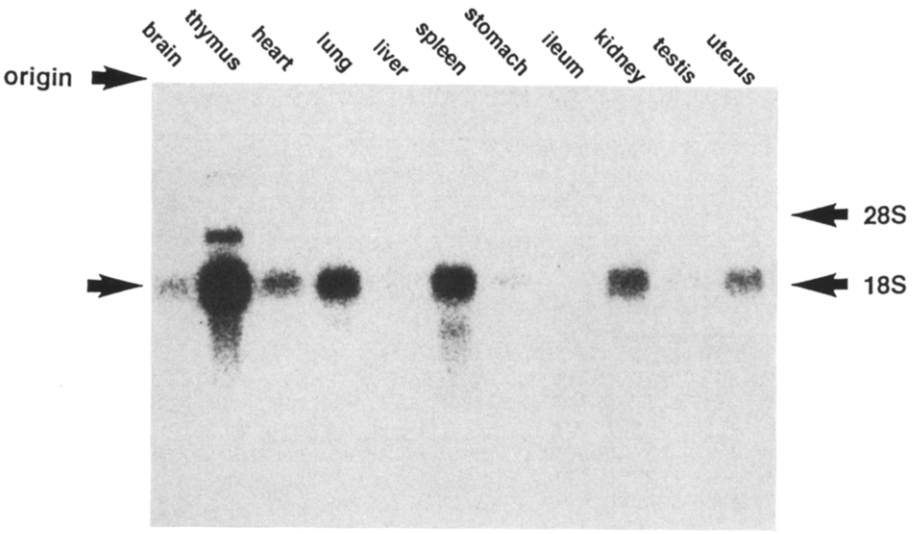

Fig.5. Northern blot analysis of various mouse organs. A left arrow indicates major bands.

yet unknown functions and roles in these tissues. Thus, importance of TXA_2 on immunological system should be re-evaluated. Kidney also shows significant amount of the TXA_2 receptor mRNA expression. TXA_2 is known to act on glomerular mesangial cells (25,26) and to regulate glomerular filtration ratio (27), and has been suggested to participate in several renal diseases such as lupus nephritis (28) and ureteral obstruction (29). Although the significant amount of the mRNA expression was observed in brain, the presence of the TXA_2 receptor in this tissue has not yet been reported. Modulation of presynaptic neurotransmitter release by TXA_2 has been suggested in peripheral nervous system such as in canine trachea (30), and in rabbit vas deferens (31). These results indicate similar functions of this compound in central nervous system. We are now investigating localization of the TXA_2 receptor in mouse brain by *in-situ* hybridization.

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