Volume 318, number 1, 91–94 FEBS 12140 February 1993

Expression of human thromboxane synthase using a baculovirus system

Chieko Yokoyama[a], Atsuro Miyata[a], Kenji Suzuki[b], Yoshiki Nishikawa[b], Tanihiro Yoshimoto[c], Shozo Yamamoto[c], Rolf Nüsing[d], Volker Ullrich[d] and Tadashi Tanabe[a]

[a]*Department of Pharmacology, National Cardiovascular Center Research Institute, Suita, Osaka 565, Japan,* [b]*Bio-Organics Research Department, International Research Laboratories, Ciba-Geigy (Japan), Takarazuka, Hyogo 665, Japan,* [c]*Department of Biochemistry, Tokushima University School of Medicine, Kuramoto-cho, Tokushima 770, Japan* and [d]*Faculty of Biology, PO Box 5560, University of Konstanz, D-7750 Konstanz, Germany*

Received 5 January 1993

Human thromboxane (TX) synthase (EC 5.3.99.5) was produced by the baculovirus expression system using cDNA encoding human TX synthase [(1991) Biochem. Biophys. Res. Commun. 78, 1479–1484]. A recombinant baculovirus TXS7 was expressed in *Spodoptera frugiperda* Sf9 insect cells. The expressed protein was recognized by monoclonal antibody, Kon 7 raised against human TX synthase [(1990) Blood 76, 80–85]. The recombinant TX synthase catalyzed the conversion of prostaglandin (PG) H_2 to TXA_2 and 12-hydroxy-heptadecatrienoic acid (HHT). Both conversions of PGH_2 to TXA_2 and HHT by the expressed TX synthase were completely inhibited by a specific TX synthase inhibitor, OKY-046 (5 μM).

Thromboxane synthase; Expression; Baculovirus

1. INTRODUCTION

Thromboxane (TX) synthase catalyzes the conversion of prostaglandin (PG) H_2 to TXA_2 which has potent biological activities as a mediator of platelet aggregation or vasoconstriction [1–3]. TX synthase has been purified from human platelets [4,5] and porcine lung [6], and the properties of the enzymes have been characterized. The molecular masses of human and porcine enzymes were reported to be 60.5 [7] and 56 kDa [6], respectively. The enzyme contains 1 mol of heme per mol of the enzyme polypeptide, and a close analogy to the group of cytochrome P450 proteins was established by optical and EPR spectroscopy.

Recently, we succeeded in cDNA cloning of human platelet TX synthase, and showed that the primary structure of the enzyme indicates a homology to those of the enzymes in cytochrome P_{450} IIIA family, especially to that of nifedipine oxidase [7]. The amino acid sequence constituting the heme-binding site of cytochrome P_{450} is well conserved in the deduced amino acid sequence of TX synthase [7]. On the other hand, Ohashi et al. also have cloned the cDNA for the enzyme from human lung cDNA library [8].

The enzymatic and protein chemical characterizations of TX synthase have been limited due to the difficulty in purification of the enzyme. In order to make more detailed studies on the structure and reaction mechanism of TX synthase, it is essential to establish an expression system for TX synthase. In this paper we report the expression of TX synthase cDNA using the baculovirus expression system and partial characterization of the expressed TX synthase.

Correspondence address. T. Tanabe, Department of Pharmacology, National Cardiovascular Center Research Institute, 5-7-1 Fujishirodai, Suita, Osaka 565, Japan. Fax: (81) (6) 872-7485.

Abbreviations: TX(A), thromboxane (A); PG, prostaglandin; HHT, 12-hydroxy-heptadecatrienoic acid; Sf9 cells, *Spodoptera frugiperda* Sf9 cells; AcNPV, *Autographa californica* nuclear polyhedrosis virus; OKY-046, sodium (*E*)-3-[4-(1H-imidazole-1-ylmethyl)phenyl]-2-propenoate; EPR, electron paramagnetic resonance; TBS, Tris-HCl (pH 7.4)-buffered saline.

2. MATERIALS AND METHODS

2.1. *Materials*

Spodoptera frugiperda Sf9 cells and baculovirus transfer vector pVL1393 were obtained from Invitrogen. Grace's insect cell culture medium was the product of Gibco. [1-^{14}C]Arachidonic acid (1.85 GBq/mmol) was purchased from Amersham. OKY-046, authentic PGE_2, $PGF_{2\alpha}$ and TXB_2 were given from ONO Pharmaceutical Co. (Osaka). All other reagents for construction of the recombinant baculovirus were described previously [7,9].

2.2. *Construction of baculovirus transfer vector of TX synthase (TXS726)*

The human TX synthase cDNA [7] was inserted into the *Bam*HI and *Xba*I sites of pVL1393 transfer vector. pHPTXS6 was digested with *Sph*I and ligated with *Xba*I linker after treatment with Klenow DNA polymerase I. The *Eco*RI (site 653)–*Xba*I fragment (1,064 bp) of the cDNA prepared from pHPTXS6 and the *Sac*I–*Eco*RI fragment (sites 210–652) from pHPTXS2 were ligated and inserted into *Sac*I and *Xba*I sites of pBluescript II SK(−)(pHPTXS26). The *Bam*HI–*Sac*I (site 209) fragment (227 bp) containing 8 bp of multicloning site of the pBluescript II was prepared from pHPTXS7, which was obtained by cloning into pBluescript II the polymerase chain reaction product with the primers 4 and 15 as described previously [7]. This *Bam*HI–*Sac*I

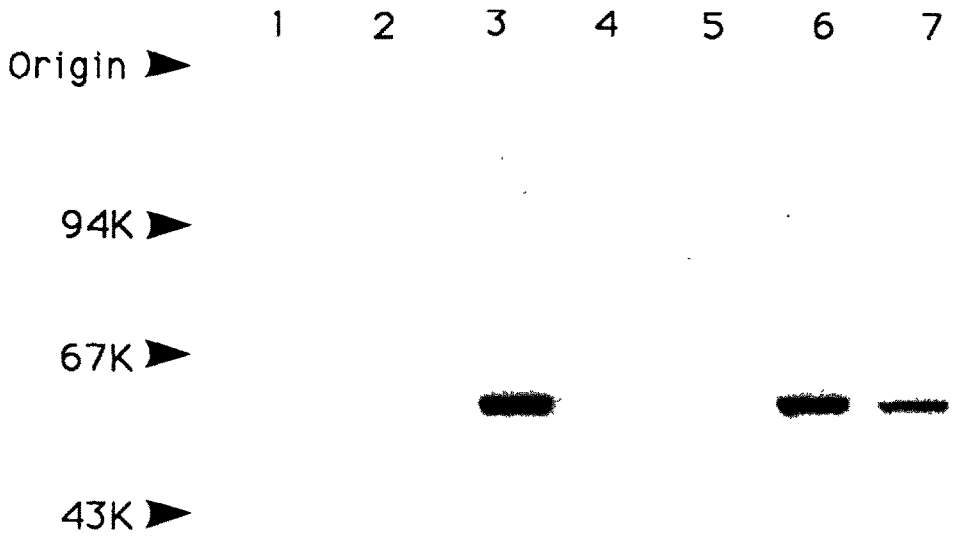

Fig. 1. Western immunoblot analysis of the expressed TX synthase in Sf9 cells. Microsomal fractions were prepared from baculovirus-infected Sf9 cells treated with (lanes 1–3) or without (lanes 4–6) 10 μM hemin as described in section 2. Microsomes from human platelets were also isolated by the same procedure. The microsomal fractions (25 μg of protein) were subjected to western immunoblot analysis using monoclonal antibody (Kon 7) against human platelet TX synthase. Lanes 1 and 4, mock-infected cells; lanes 2 and 5, wild-type AcNPV-infected cells; lanes 3 and 6, TXS7-infected cells; lane 7, human platelets.

fragment and the *Sac*I–*Xba*I fragment (1,507 bp) from pHPTXS26 were ligated and inserted into the *Bam*HI and *Xba*I sites of the pVL1393 transfer vector (TXS726). The construct of each recombinant plasmid was confirmed by the restriction mapping or DNA sequencing analysis.

2.3. *Baculovirus expression system*

Sf9 cells were maintained in monolayer culture in Grace's insect medium supplemented with 10% fetal bovine serum, 0.33% yeastolate and 0.33% lactalbumin hydrolysate at 27°C [10]. To produce a recombinant virus, Sf9 cells (1.5×10^6 cells) was transfected with a mixture of the recombinant plasmid TXS726 (50 μg) and wild-type baculovirus DNA (AcNPV; 1 μg) by the calcium-phosphate precipitation method. A recombinant baculovirus TXS7 was isolated by the combination of plaque assay and slot blot hybridization using ^{32}P-labeled cDNA fragments of TX synthase as probes, and amplified for further use. Sf9 cells were infected with a wild-type (AcNPV) or the recombinant (TXS7) baculovirus. At 3 days after infection, the cells were harvested (2×10^8 cells) and incubated for 5 h with or without 10 μM hemin in the serum-supplemented medium. The cells were washed with phosphate-buffered saline and stored at −80°C. The microsomal fractions of the cells were prepared according to the method of Haurand and Ullrich [4]. The cells (2×10^8 cells) were homogenized in 20 ml of 10 mM potassium phosphate buffer (pH 7.0), 10 mM EDTA, 5 mM glucose, 0.1 mM dithiothreitol (DTT), 1.15% KCl, 2 μg/ml leupeptin, 2 μg/ml pepstatin, 10 μg/ml soybean trypsin inhibitor, and 44 μg/ml phenylmethylsulfonyl fluoride, and sonicated 4 times with a Branson sonifier model 450 for 30 s. The homogenate was centrifuged at $7,000 \times g$ for 15 min and the supernatant was centrifuged at $105,000 \times g$ for 60 min. Pellets were suspended in 3 ml of 10 mM potassium phosphate buffer (pH 7.0) containing 20% glycerol, 1 mM DTT and 1 mM EDTA by sonication. Protein concentration was determined by the method of Lowry [11] using bovine serum albumin as a standard, and adjusted to 5 mg/ml with the same solution for immunoblot analysis and TX synthase assay.

2.4. *Western immunoblot analysis*

Microsomal fractions from infected Sf9 cells and human platelet were subjected to 10% SDS-PAGE by the method of Laemmli [12]. The proteins were transferred electrophoretically onto a polyvinylidene difluoride (PVDF) membrane (Immobilon, Millipore) according to the method of Towbin et al. [13]. After pretreatment with 10% horse serum in Tris-HCl-buffered saline (TBS), pH 7.4, at room temperature for 30 min, the blotted membrane was incubated with the monoclonal antibody against human platelet TXA synthase, Kon 7 [14,15] in TBS containing 3% skim milk. After rinsing with TBS containing 0.05% Tween 20, the membrane was incubated with anti-mouse IgG horse antibody conjugated with horseradish peroxidase (Vector Laboratories) in TBS containing 3% skim milk at 37°C for 30 min. After successive washings with TBS containing 0.05% Tween 20 and then with TBS, immunoreactive bands were detected by the Immunostaining HRP kit (Konica, Tokyo).

2.5. *TX synthase assay*

[1-^{14}C]PGH_2 was prepared from [1-^{14}C]arachidonic acid using microsomes of sheep vesicular glands, and TX synthase assay was carried out as described in [16]. Briefly, the standard reaction mixture for TX synthase assay contained 0.1 M Tris-HCl buffer at pH 7.4, [1-^{14}C]PGH_2 (80,000 cpm/5 nmol dissolved in 5 μl of acetone) and an enzyme preparation in a final volume of 100 μl. The reaction was carried out at 24°C for 1 min and terminated by the addition of 0.3 ml of a mixture of ethyl ether/methanol/0.2 M citric acid (30:4:1). The organic phase (100 μl) was directly applied on a silica gel plate at 4°C, and the plate was developed at room temperature using an organic layer of ethyl acetate/2,2,4-trimethylpentane/acetic acid/water (110:50:20:100). The radioactivity of the plates was monitored using

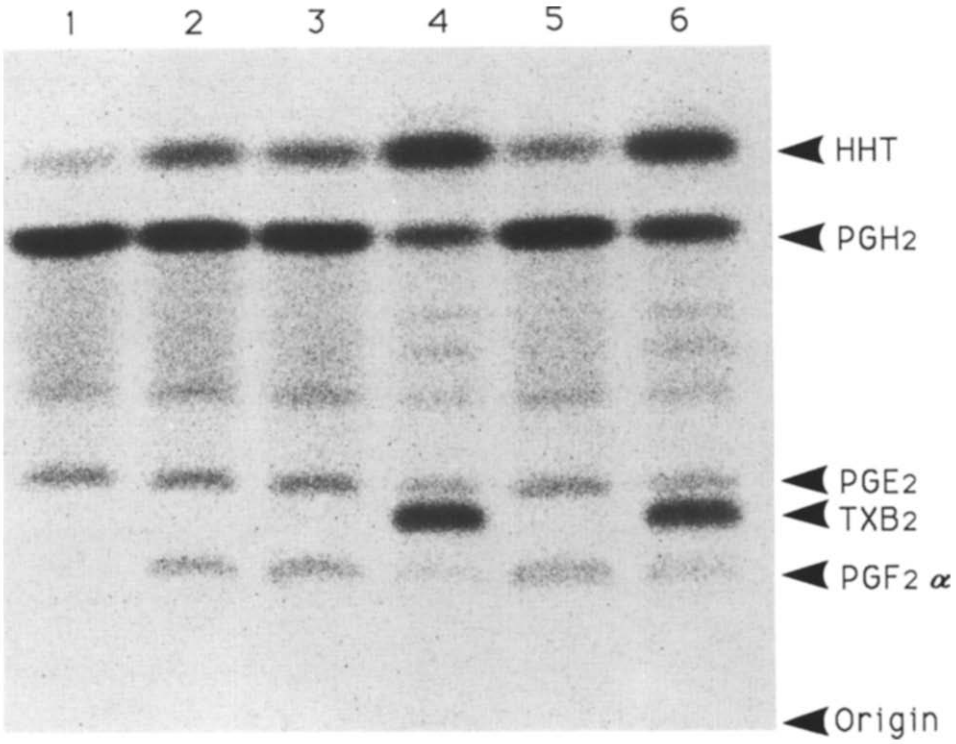

Fig. 2. Formation of TXB_2 and HHT from PGH_2 by microsomes from infected Sf9 cells. Microsomal fractions were prepared from the baculovirus-infected Sf9 cells treated with (lanes 2–5) or without (lane 6) 10 μM hemin as described in Fig. 1. Microsomal fractions (5 μg of protein) were incubated with 50 μM [1-^{14}C]PGH_2 at 24°C for 1 min in the presence (lane 5) or absence (lanes 2–4 and 6) of 5 μM OKY-046 as described in section 2. The reaction products were separated by thin-layer chromatography and radioactive products were detected by a Fujix Bio-image analyzer BAS 2000. Authentic TXB_2, PGE_2 and $PGF_{2\alpha}$ were also run. Migrations of PGH_2, TXB_2, HHT, PGE_2 and $PGF_{2\alpha}$ are indicated by the arrowheads. Lane 1, no enzyme preparation; lane 2, mock-infected cells; lane 3, wild-type virus (AcNPV)-infected cells; lanes 4-6, TXS7-infected cells.

either an Imaging Scanner System 400 (Packard) or a Fujix BAS 2000 Bio-imaging analyzer (Fuji Photo Film Co., Tokyo).

3. RESULTS AND DISCUSSION

It has been shown by optical and EPR spectroscopic studies on human platelet TX synthase that the enzyme is a cytochrome P_{450}-like protein [4,5]. This finding has been confirmed by cDNA cloning of the enzyme. Namely, the primary structure of the enzyme deduced from the nucleotide sequence of the cDNA for the enzyme has a homology with those of cytochrome P_{450}s. Especially, the sequence around the cysteine (residue 479) which probably constitutes the fifth ligand of heme exhibits a highly conserved sequence [7,8].

The study on TX synthase, however, has been restricted due to the difficulty in obtaining sufficient amount of the enzyme. Thus, we tried to express TX synthase with our cloned human TX synthase cDNA using the baculovirus expression system. Recently, many investigators succeeded in expression of membrane proteins by the baculovirus expression system. Several cytochrome P_{450}s were also expressed by this system. Asseffa et al. reported that exogenous hemin added in the culture medium increased the enzyme activity of cytochrome P_{450} IIA1 by conversion of the expressed apoenzyme to the holoenzyme, and the baculovirus expression system can be used as a system to produce high levels of mammalian cytochrome P_{450}s containing custom modified heme moieties [17].

For expression of the recombinant TX synthase, a recombinant baculovirus (TXS7) with an insert of the entire protein-coding region of human TX synthase gene was constructed and expressed in Sf9 cells. Previously, Nüsing et al. prepared several polyclonal and monoclonal antibodies against human platelet TX synthase and characterized [14,15]. Western immunoblot analysis of the expressed protein in Sf9 cells was carried out using the monoclonal antibody Kon 7. TX synthase was purified from microsomal fractions [4,5], and Ohta et al. showed by immunoelectronmicroscopy that the expressed cytochrome P_{450} F1 was linked to the endoplasmic reticulum of the recombinant virus-infected Sf9 cells [18]. Thus, microsomal fractions of the infected cells were subjected to immunoblot analysis. As shown in Fig. 1, an immunoreactive band appeared in the microsomal fraction from TXS7-infected cells (lanes 3 and 6). The positive band migrated at a molecular mass of approximately 58 kDa and was indistinguishable from human platelet TX synthase (lane 7). No immunoreactive band was found in the mock or wild-type AcNPV-infected cells (lanes 1, 2, 4 and 5). These results suggest

that the human TX synthase protein was expressed in TXS7-infected cells. The ratio of immunochemical signals of TX synthase from TXS7-infected cells and human platelets was about 2:1. The intensity of the immunoreactive band from the cells incubated in the absence of exogenous hemin for 5 h before collection of cells was practically the same with that of the cells incubated in the presence of hemin (lanes 3 and 6). Thus, the treatment with hemin did not influence the synthesis of TX synthase apoprotein as found in the expression of other cytochrome P_{450}s [17,18].

To examine the TX synthase activity of the recombinant protein, the microsomal fractions from the infected cells were incubated with ^{14}C-labeled PGH_2 as a substrate and the radioactive products were separated by silica gel thin layer chromatography (Fig. 2). When the microsomal fraction from TXS7-infected cells incubated in the culture medium containing 10 μM hemin for 5 h was incubated with PGH_2, PGH_2 was converted to HHT and TXB_2 which is a stable degradation product of TXA_2 (Fig. 2, lane 4). The TXB_2-forming activity of the microsome was 130 nmol/min/mg protein, which is about twice as much as that of human platelet microsome [4]. Furthermore, the production of TXB_2 and HHT was completely inhibited by 5 μM OKY-046 that is a specific inhibitor for TX synthase [19] as shown in Fig. 3 (lane 5). These results suggest that Sf9 cells infected with TXS7 have expressed an active TX synthase. But, the purified human enzyme produced TXB_2 and HHT from PGH_2 with a ratio of 1:1 [4,5]. The ratio of TXB_2 and HHT formed by the microsomal fraction from TXS7-infected cells was approximately 1:1.8. The discrepancy may be ascribed to crude enzyme preparations used for the enzymatic reactions such as microsomes [16]. The treatment of the TXS7-infected cells with hemin for 5 h did not affect the increase in the TX synthase activity. This result suggests that most of the expressed TX synthase protein existed as the holoenzyme probably due to the sufficient supply of heme in the Sf9 cells at the level of expression of TX synthase in this study.

We are now attempting to express a larger amount of TX synthase for detailed studies on the structure and reaction mechanism of TX synthase. The site-directed mutagenesis study is also now in progress.

Acknowledgements. We thank Ms. T. Sugimoto, S. Bando and R. Tsuchiya for their technical assistance. This work was supported in part by grants from the Ministry of Health and Welfare, the Ministry of Education, Science and Culture of Japan, and Japan Cardiovascular Research Foundation. This research was also supported in part by the Deutsche Forschungsgemeinschaft.

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