Arg^{60} to Leu Mutation of the Human Thromboxane A_2 Receptor in a Dominantly Inherited Bleeding Disorder

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Abstract

Recent advances in molecular genetics have revealed the mechanisms underlying a variety of inherited human disorders. Among them, mutations in G protein–coupled receptors have clearly demonstrated two types of abnormalities, namely loss of function and constitutive activation of the receptors. Thromboxane A_2(TXA_2) receptor is a member of the family of G protein–coupled receptors and performs an essential role in hemostasis by interacting with TXA_2 to induce platelet aggregation. Here we identify a single amino acid substitution (Arg^{60}→Leu) in the first cytoplasmic loop of the TXA_2 receptor in a dominantly inherited bleeding disorder characterized by defective platelet response to TXA_2. This mutation was found exclusively in affected members of two unrelated families with the disorder. The mutant receptor expressed in Chinese hamster ovary cells showed decreased agonist-induced second messenger formation despite its normal ligand binding affinities. These results suggest that the Arg^{60} to Leu mutation is responsible for the disorder. Moreover, dominant inheritance of the disorder suggests the possibility that the mutation produces a dominant negative TXA_2 receptor. (*J. Clin. Invest.* 94:1662–1667.) Key words: G protein • blood platelets • phospholipase C • human genetics • point mutation

Introduction

A large number of G protein–coupled receptors have been identified and shown to induce their intracellular responses via coupling to different classes of guanine nucleotide-binding proteins (G proteins).[1] Given that numerous physiologically important functions are mediated by G protein–coupled receptors, it is expected that abnormalities in G protein–coupled receptors can underlie a variety of human diseases. Indeed, several mutations in G protein–coupled receptors have been shown to be associated with human genetic diseases, which result from either loss of function or constitutive activation of the affected receptors (see references 1–3 for reviews).

Loss of function of a G protein–coupled receptor has been typically shown in congenital nephrogenic diabetes insipidus, an X-linked recessive disorder in which mutations in the V2 vasopressin receptor are responsible for the resistance to the antidiuretic action of the hormone in the kidney (4, 5). Because the gene for the receptor is on the X chromosome, any loss of function mutation results in the clinically apparent disease in males. On the other hand, some mutations have been shown to cause constitutive activation of the luteinizing hormone receptor in familial male precocious puberty (6) and rhodopsin in a severe form of retinitis pigmentosa (7). These constitutively active mutations are sure to produce a dominant phenotype; i.e., the diseased state is inherited in a dominant manner. Loss of function mutations can also lead to a dominant phenotype, if half of the gene product is not sufficient for normal function. The other mechanism that has not been identified clearly yet but can potentially cause a genetic disease is a dominant negative mutation, which, if genetically encoded, should manifest itself as a defective function of the receptor with a dominant inheritance.

We have previously cloned the human TXA_2 receptor and shown that it belongs to the superfamily of G protein–coupled receptors (8). It mediates the aggregation of platelets to TXA_2, a major arachidonic acid metabolite, by activating the phospholipase C (PLC) cascade via a G protein–coupled pathway (9–11). The physiological importance of the TXA_2-mediated signaling pathway has been demonstrated in a group of patients whose hemostatic defects were found to be associated with platelet unresponsiveness to TXA_2 (12–19). Recently, we have reported two unrelated patients with a mild bleeding disorder whose platelets showed impaired aggregation responses to TXA_2 and its analogues, despite the normal response to thrombin (20, 21). Although the patients' platelets exhibited normal binding activities to TXA_2 analogues, they showed decreased GTPase activity and second messenger formation when stimulated by 9,11-epithio-11,12-methano-TXA_2 (STA_2), a stable TXA_2 agonist (20–22). These findings led us to speculate that the defect in the patients is due to impaired coupling of the

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1. *Abbreviations used in this paper:* CHO, Chinese hamster ovary; G protein, guanine nucleotide-binding protein; IP_3, inositol 1,4,5-triphosphate; PI, phosphatidylinositol; PLC phospholipase C; PPP, platelet-poor plasma; PRP, platelet-rich plasma; RT, reverse transcription; S-145, 5Z-7-(3-*endo*-phenylsulphonylamino bicyclo [2.2.1] hept-2-*exo*-yl) heptenoic acid; STA_2, 9,11-epithio-11,12-methano-TXA_2.

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TXA_2 receptor to the G protein, which is caused by an abnormality of the TXA_2 receptor itself.

Here we demonstrate a mutation in the first cytoplasmic loop of the TXA_2 receptor exclusively in affected members of two unrelated families with the disorder. Consistently, the mutant receptor expressed in Chinese hamster ovary (CHO) cells shows unaltered ligand binding affinities but decreased STA_2-induced activation of PLC, when compared to the wild-type receptor. Moreover, we show that this defect of the platelets is inherited dominantly.

Methods

Subjects. Two unrelated patients T.T. (20, 22) and Y.O. (21) and family members of the patient T.T. were studied. Informed consent was obtained from all subjects.

Materials. STA_2 was a generous gift from Ono Pharmaceutical Co. Ltd. (Osaka, Japan). S-145 [5Z-7-(3-*endo*-phenylsulphonylamino bicyclo [2.2.1] hept-2-*exo*-yl) heptenoic acid] and its tritium derivative [^{3}H]S-145 were generous gifts from Shionogi Research Laboratories (Osaka, Japan). *Myo*-[2-^{3}H]inositol (19.1 Ci/mmol) was obtained from Amersham Corp (Arlington Heights, IL). All other materials were obtained from the sources as described previously (20).

Platelet aggregation study. Platelet-rich plasma (PRP) and platelet-poor plasma (PPP) were prepared from the family members and control subjects, as described previously (20). Platelet concentration of PRP was adjusted to 3×10^8 platelets/ml by autologous PPP. Washed platelet suspensions were prepared as previously described (20). The concentration of the washed platelets was also adjusted to 3×10^8 platelets/ml. The aggregation studies were performed in PRP for STA_2-, ADP-, and epinephrine-induced aggregation, and in washed platelets for thrombin-induced aggregation. The aggregation was monitored essentially by the method of Born (23). The PRP or washed platelet suspension was placed in a tube, and the increase in light transmission was followed at 37°C in a dual-channel aggregometer (NKK Hematracer 1 model PAT-2A; Niko BioScience Co., Tokyo, Japan). The light transmission of PRP or washed platelet suspension was set at 0% and that of PPP or buffer at 100%.

Amplification of platelet mRNA. Total RNA was extracted from platelets of healthy volunteers and the patients (T.T. and Y.O.) by the acid guanidium-phenol-chloroform (AGPC) method (24). ~ 10 μg of total RNA was recovered from the platelets of 50 ml blood sample. 1 μg of total RNA was reverse-transcribed in a volume of 20 μl (25) and amplified by PCR using primers next to the coding region of the human TXA_2 receptor (forward primer, 5′-GTCTGCAGCATCGGCCTGATG-3′; reverse primer, 5′-GAGGGGCGCTCTGTCCACTT-3′) (8). PCR was performed in a final volume of 20 μl, containing 2 μl reverse transcription (RT) reaction mixture, 50 mM KCl, 10 mM Tris-HCl (pH 8.8), 1.5 mM $MgCl_2$, 0.1% Triton X-100, 10% dimethylsulfoxide, 0.25 mM dNTPs, 1 U Taq Polymerase (Wako Pure Chemical Industries Ltd., Osaka, Japan), and 20 pmol of each primer. After denaturation at 94°C for 2 min, 35 cycles (94°C for 1 min, 64°C for 1 min, 72°C for 1.5 min) were followed by a final elongation step for 3.5 min at 72°C. PCR products were electrophoresed, excised, purified using Geneclean II (BIO 101, Inc., Vista, CA), and blunt-end–ligated to pBluescript II SK+ (Stratagene, LaJolla, CA). Plasmid subclones were sequenced by the dideoxy method.

Genomic studies. Genomic DNA was prepared from white blood cells of the patients (T.T. and Y.O.), family members of the patient T.T., and control subjects as described (26). A 320-bp fragment was amplified by PCR, using primers 5′-ATGTGGCCCAACGGCAGTTC-3′ and 5′-AAGCGACAGAGACGGCAGCC-3′. PCR was done as described in RT-PCR except that 0.1 μg genomic DNA was used instead of RT reaction mixture as a template and that the annealing temperature was lowered to 62°C. PCR products were purified from a 2% agarose gel, digested with HhaI (TOYOBO Co. Ltd., Osaka, Japan), separated on a 10% polyacrylamide gel, and visualized with ethidium bromide.

Expression of wild-type and mutant TXA_2 receptors. cDNAs encoding the wild-type and mutant TXA_2 receptors were inserted into a mammalian expression vector, pEF-BOS (27) with a blasticidin S-resistance marker (Funakoshi Co. Ltd., Tokyo, Japan) and introduced into the CHO cells by the calcium phosphate method (28). Stable transformants were cloned by selection in the α-modified minimal essential medium containing 10% FCS and 15 ng/ml blasticidin S (Funakoshi Co. Ltd.). For ligand binding assay, cells were cultured in 15 cm dishes to confluency and harvested as described previously (29). Cells were washed once and suspended at 1×10^7 cells/ml in a buffer containing 20 mM Hepes (pH 7.4), 140 mM NaCl, 5 mM KCl, and 5 mM $MgCl_2$. Binding assays were performed as described (30). For phosphatidylinositol (PI) turnover, cells were cultured in six-well plates to 90% confluency in the medium described above and then incubated for 20 h in inositol-free DME with 10% dialyzed FCS containing 1 μCi/ml *myo*-[2-^{3}H]-inositol. The cells were washed with Hepes-buffered saline and preincubated with 10 mM LiCl in Hepes-buffered saline at 37°C for 10 min. Then various concentrations of STA_2 were added to each medium. After 5 min incubation, the medium was removed and the reaction was terminated by 1 ml of 5% TCA. The generation of inositol 1,4,5-triphosphate (IP_3) was measured as described (31).

Results

Identification of a family with defective platelet response to TXA_2. We previously described a patient (T.T.) with a mild bleeding disorder characterized by defective platelet response to TXA_2 (20, 22). The patient's platelets showed defective aggregation response to STA_2 (Fig. 1 *A*), although they responded normally to thrombin (Fig. 1 *B*). To determine whether this platelet disorder is hereditary, platelet functions of five additional family members were studied. The patient's daughter showed a similar but slightly milder defect in platelet aggregation response to STA_2 (Fig. 1 *C*). The son and the brother of the patient showed similar impairments of aggregation to that of his daughter (data not shown). On the other hand, his sister showed normal response (Fig. 1 *C*), and so did his wife (data not shown). The aggregation responses to 2 μM ADP and 5 μM epinephrine in the patient's daughter (Fig. 1, *D* and *E*), as well as his brother and son (data not shown), were suppressed. Again, these results indicate a defect in platelet responsiveness to TXA_2, since TXA_2 serves to amplify the platelet response to weak agonists such as ADP and epinephrine. There was no history of consanguinity. Thus, this platelet disorder appears to be transmitted as an autosomal dominant trait, probably due to a defect in the TXA_2 receptor gene. We therefore decided to search for a mutation in the TXA_2 receptor.

Identification of a mutation in the TXA_2 receptor. To search for a mutation in the TXA_2 receptor that might cause the bleeding disorder in this family, the entire coding region of the TXA_2 receptor cDNA (8) was amplified by the RT-PCR method from the patient (T.T.) platelet RNA, and the PCR products were subcloned and sequenced. All the clones sequenced (more than 10 clones) contained a single base change (G→T) at nucleotide 179 (Fig. 2 *A*), resulting in the substitution of a leucine (CTC) for the arginine (CGC) at the 60th amino acid which is located in the first cytoplasmic loop of the TXA_2 receptor (Fig. 2 *B*). Arg^{60} corresponds to a highly conserved basic residue among G protein–coupled receptors (Fig. 2 *C*). This mutation was not detected in cDNA fragments generated from normal individuals, thus excluding the possibility of a polymorphism. These results indicate that either the patient is homozygous for this mutation or only the mutated allele is expressed.

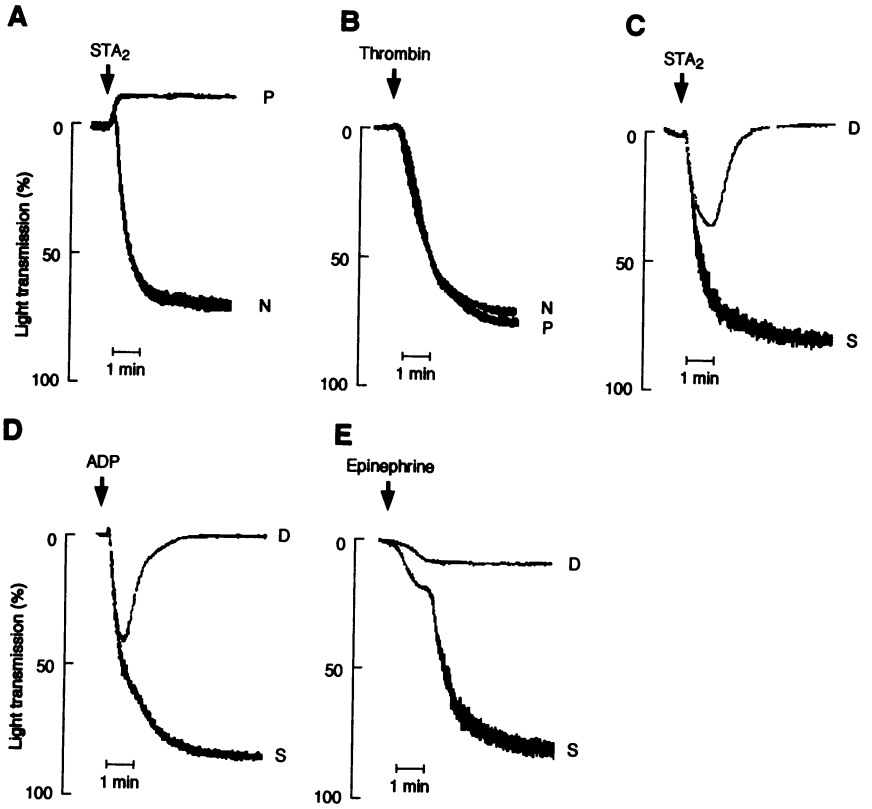

Figure 1. Representative platelet aggregation tracings of the patient (T.T.) (*A* and *B*) and his family members (*C–E*). Arrows indicate the addition of the aggregating agent. (*A*) The response of the platelets from the patient (*P*) and a normal subject (*N*) to 2 μM STA_2. The platelets of the patient showed no aggregation in response to STA_2. (*B*) The response of the platelets from the patient (*P*) and a normal subject (*N*) to 0.05 U/ml thrombin. Both platelets aggregated equally well in response to thrombin. (*C–E*) The response of the platelets from the patient's daughter (*D*) and sister (*S*) to 2 μM STA_2 (*C*), 2 μM ADP (*D*), and 5 μM epinephrine (*E*). A similar but slightly milder defect in platelet aggregation was observed in his daughter, but not in his sister. The son and the brother of the patient showed similar impairments of aggregation to that of his daughter, while his wife showed normal aggregation (data not shown).

Genomic analyses of family members. Because the mutation destroys a HhaI restriction enzyme digestion site (GCGC), the mutation can be detected more easily by analyzing the HhaI digestion pattern of PCR products. As shown in Fig. 3 *A*, the G→T transition identified here destroys the third HhaI site of PCR products, producing the 117 bp fragment which is larger than the wild-type 94 bp fragment when PCR products are digested with HhaI. Genomic analysis by this method demonstrated that the patient (T.T.) was indeed homozygous and his brother and two children were heterozygous for the mutation (Fig. 3 *B*). Consistently, his wife and sister had no mutation in either allele. In each case, the accordance of the expression of either the normal or the mutated allele to the genotype was confirmed by RT-PCR followed by HhaI digestion (data not shown). No amino acid mutation was encoded in the normal allele of heterozygotes. These analyses of the family members showed a complete correspondence of the mutant allele with the impairment in platelet aggregation. Furthermore, another patient (Y.O.) from an unrelated family with a similar defect in platelet functions (21) was analyzed in the same way and found to have an identical mutation (data not shown). The occurrence of the same Arg^{60} to Leu mutation in two unrelated patients strongly suggests that this mutation causes the disorder.

Functional properties of the expressed mutant TXA_2 receptor. To clarify the effect of the Arg^{60} to Leu mutation on the receptor function, the wild-type and mutant TXA_2 receptors were stably expressed in CHO cells for investigation of functional properties. Stable transformants expressing either the wild-type or mutant receptors were identified by binding assays. Cell lines expressing a similar number of wild-type or mutant receptors were chosen for further experiments. The mutant receptor exhibited an unaltered binding affinity for a radiolabeled TXA_2 analogue [^{3}H]S-145, compared to that of the wild-type receptor (Fig. 4 *A*). The specific [^{3}H]S-145 binding to wild-type and mutant receptors was displaced similarly by STA_2 (K_i = 12.0 and 13.3 nM for the wild-type and mutant receptors, respectively). These findings are consistent with the observation that the patients' platelets have normal binding activities to TXA_2 analogues (20–22). Then, both cell lines were examined for the activation of the PLC cascade. In the cells expressing the wild-type TXA_2 receptor, STA_2 produced a concentration-dependent increase in IP_3 production with a maximal stimulation of 6.3-fold (Fig. 4 *B*). In contrast, the mutant TXA_2 receptor produced only a twofold increase in IP_3 production (Fig. 4 *B*). The responses to thrombin (acting through endogenous receptors) were similar in both cell lines (40% and 42% increase in IP_3 production for the wild-type and mutant receptors, respectively). These results indicate that the mutant receptor is impaired in its ability to produce agonist-induced PI hydrolysis. Thus, the expression of the mutant receptor resulted in the functional abnormality equivalent to that associated with the patients.

Discussion

We have shown that a mutation in the TXA_2 receptor is associated with a bleeding disorder characterized by defective platelet response to TXA_2. This mutation was identified in two unrelated patients with the disorder, who showed a similar defect in platelet responsiveness to TXA_2 (Fig. 1, *A* and *B;* references 20–22). Their platelets exhibit impaired second messenger formation induced by a TXA_2 agonist despite normal ligand binding activities (20–22). Consistently, the mutant receptor expressed in CHO cells exhibited unaltered binding affinities for TXA_2 ana-

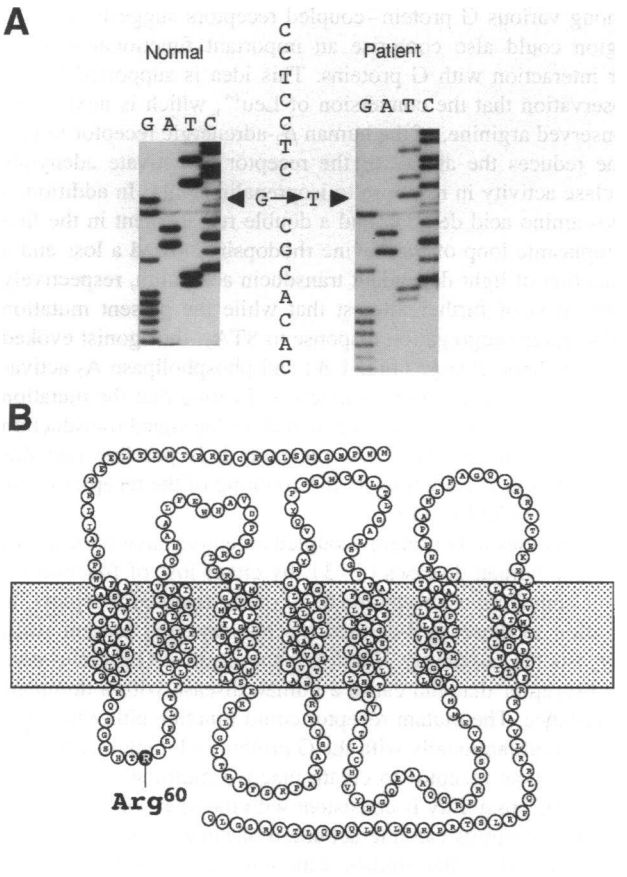

Figure 2. Missense mutation in the TXA_2 receptor of the patient (T.T.). (*A*) Comparison of the nucleotide sequences of the TXA_2 receptor cDNAs between the patient and a normal subject. Portions of the sequence gels of cDNAs from the patient (*right*) and a normal subject (*left*) are shown. All clones sequenced from the patient RNA showed a G→T transition at nucleotide 179. This mutation encodes a substitution of Leu for Arg^{60}. (*B*) Schematic structure of the TXA_2 receptor. The position of Arg^{60} is indicated. (*C*) Comparison of amino acids in the first cytoplasmic loop among representative G protein–coupled receptors. Note that a basic residue, Arg or Lys, is conserved in the middle of the first cytoplasmic loop among the members of the G protein–coupled receptor superfamily. Protein sequences of the first cytoplasmic loop of human m_3 muscarinic receptor (34), human α_{2c}-adrenergic receptor (35), human β_1-adrenergic receptor (36), human substance P receptor (37), and human TSH receptor (38) are included. Conserved basic residues are boxed.

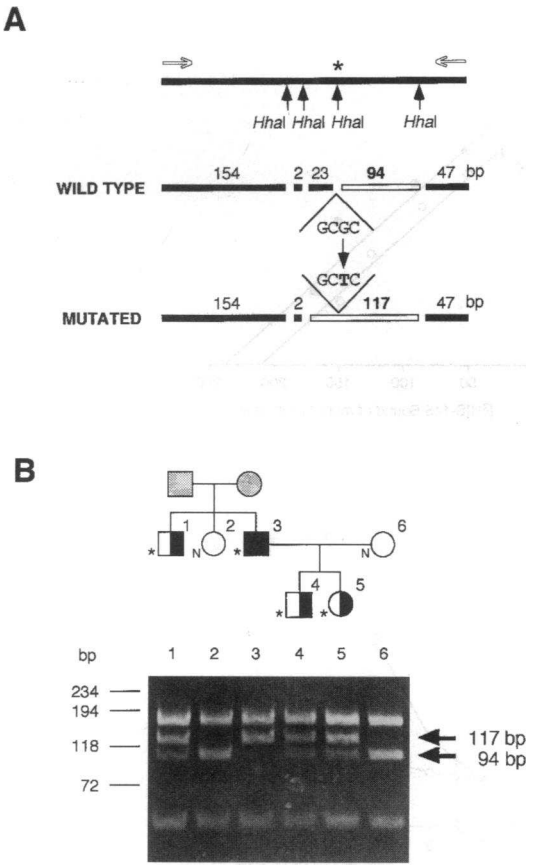

Figure 3. Genomic analysis of the family members. (*A*) Schematic representation of method for detecting the Arg^{60} to Leu mutation. PCR primers (*open arrows*) are designed to amplify a 320 bp fragment which has four *Hha*I sites (*closed arrows*). The G→T transition destroys the third *Hha*I site (*asterisk*). Consequently, the wild-type PCR product produces five restriction fragments of 154, 2, 23, 94, and 47 bp, whereas the mutated PCR product produces four fragments of 154, 2, 117, and 47 bp. The diagnostic fragments of 94 bp (wild-type) and 117 bp (mutated) are indicated as open bars. (*B*) *Hha*I restriction enzyme analysis of DNA from the family members. The patient (lane *3*) shows the homozygous digestion pattern for the mutation. His brother (lane *1*), son (lane *4*), and daughter (lane *5*) show the heterozygous digestion pattern. His sister (lane *2*) and wife (lane *6*) have no mutation in either allele. Size marker positions (ΦX174 digested by *Hae*III) are indicated to the left of lane 1. These results are illustrated in the pedigree at the top; a solid symbol designates a homozygote and half-solid symbols heterozygotes. The patient's parents are deceased and not available for study (*dotted symbols*). Squares and circles indicate males and females, respectively. The status of platelet aggregation is also shown; individuals whose platelets showed abnormal aggregation responses are denoted by asterisks and those with normal aggregation responses are denoted by the letter N.

logues compared to the wild-type receptor, but was impaired in its ability to mediate agonist-induced stimulation of PLC (Fig. 4). Investigations of family members of one patient (T.T.) suggested that this disorder is inherited as an autosomal dominant trait (Fig. 1, *C–E*). Consistent with its autosomal inheritance, the TXA_2 receptor gene has been mapped on a tip of the short arm of human chromosome 19 (32). Furthermore, genomic analyses revealed that the heterozygotes for the mutation demonstrate a similar, but slightly weak, phenotype of the impaired platelet aggregation to a TXA_2 agonist compared to the homozygote (Fig. 1 and Fig. 3 *B*). This may not be explained with haploid insufficiency, since it has been shown that less than half the number of receptors are sufficient for irreversible aggregation of platelets by a TXA_2 agonist (33). These results indi-

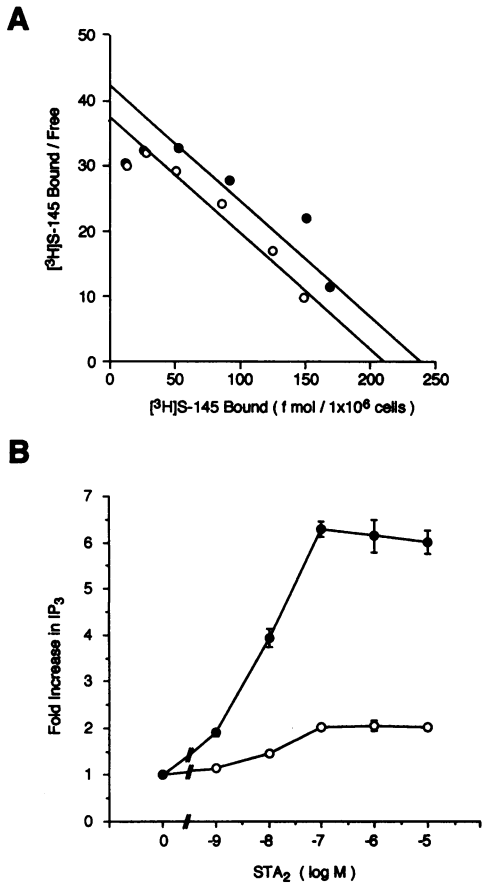

Figure 4. Functional properties of the wild-type and mutant TXA_{I2} receptors stably expressed in CHO cells. (*A*) Scatchard analyses of the wild-type (•) and mutant (○) TXA_2 receptors. The mutant TXA_2 receptor exhibited an affinity for [^{3}H]S-145, a TXA_2 antagonist, (K_d = 5.6 nM) which was comparable to that of the wild-type receptor (K_d = 5.7 nM). Both cell lines expressed a similar number of receptors (B_{max} = 240 and 210 fmol/1 × 10^6 cells for the wild-type and mutant receptors, respectively). (*B*) STA_2-induced IP_3 accumulation in CHO cells expressing the wild-type (•) and mutant (○) TXA_2 receptors. Data points are mean±SEM from three replicate wells from a single experiment. Where not shown, an SEM bar is smaller than the symbol. The data are presented as fold increases, where the values from the cells in the absence of STA_2 were chosen as the reference values. The radioactivity (cpm per well, mean±SEM, n=3) in the cells expressing the wild-type and mutant receptors without STA_2 were 622±5 and 1031±87, respectively. The results were independently confirmed in two additional experiments.

cate that the mutant TXA_2 receptor is defective in its signal transduction by itself and suppresses the function of the wild-type receptor.

Based on its sequence homology to other G protein–coupled receptors, the TXA_2 receptor is thought to have the topography represented in Fig. 2 *B*. The Arg^{60} is located in the first cytoplasmic loop (Fig. 2 *B*) and corresponds to a highly conserved basic residue among most of the G protein–coupled receptors (Fig. 2 *C*, references 34–38). The cytoplasmic domains are implicated a priori in the receptor–G protein interaction. Although the third loop and the COOH-terminal domain have been strengthened more than the first cytoplasmic loop, the conservation of the basic residue in the first cytoplasmic domain among various G protein–coupled receptors suggests that this region could also comprise an important functional element for interaction with G proteins. This idea is supported by the observation that the conversion of Leu^{64}, which is next to the conserved arginine, of the human β_2-adrenergic receptor to glycine reduces the ability of the receptor to activate adenylate cyclase activity in response to isoprenaline (39). In addition, a two–amino acid deletion and a double replacement in the first cytoplasmic loop of the bovine rhodopsin showed a loss and a reduction of light-dependent transducin activation, respectively (40). It is of further interest that while the present mutation did suppress aggregation response to STA_2, the agonist evoked platelet shape change (Fig. 1 *A*) and phospholipase A_2 activation (22) in the patients' platelets, indicating that the mutation found in this study may not abolish all of the signal transduction pathways through the TXA_2 receptor. It is possible that this mutation specifically impairs the coupling of the receptor to the G protein linked to PLC.

Mutations in G protein–coupled receptors have been shown to cause human diseases (1–3), by either loss of function (4, 5) or constitutive activation (6, 7) of the mutated receptors. In addition to these well-defined mechanisms, the present study suggests the possibility of a dominant negative G protein–coupled receptor that can cause a human disease with a dominant inheritance. The mutant receptor could function either by interacting nonfunctionally with the G protein or by interacting with the wild-type receptor to create inactive multimeric receptors. The latter possibility is consistent with the idea that G protein–coupled receptors can interact at a molecular level, as has been demonstrated in the studies with muscarinic receptors (41). The existence of muscarinic dimers has been postulated based on an analysis of the agonist-induced binding properties of muscarinic receptors (42, 43). Although further biochemical analyses will be required to elucidate the detailed mechanism of the dominant negative receptor function, the present study indicates a novel category of human disease that is caused by a dominant negative mutation of a G protein–coupled receptor.

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