Identification of Residues Important for Ligand Binding of Thromboxane A2 Receptor in the Second Extracellular Loop Using the NMR Experiment-guided Mutagenesis Approach*

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The second extracellular loop (eLP2) of the thromboxane A2 receptor (TP) had been proposed to be involved in ligand binding. Through two-dimensional 1H NMR experiments, the overall three-dimensional structure of a constrained synthetic peptide mimicking the eLP2 had been determined by our group (Ruan, K.-H., So, S.-P., Wu, J., Li, D., Huang, A., and Kung, J. (2001) Biochemistry 40, 275–280). To further identify the residues involved in ligand binding, a TP receptor antagonist, SQ29,548 was used to interact with the synthetic peptide. High resolution two-dimensional 1H NMR experiments, NOESY, and TOCSY were performed for the peptide, SQ29,548, and peptide with SQ29,548, respectively. Through completed 1H NMR assignment and by comparing the different spectra, extra peaks were observed on the NOEY spectrum of the peptide with SQ29,548, which implied the contacts between residues of eLP2 at Val176, Thr185, Thr186, and Leu187 with SQ29,548, respectively. The residues responsible for specific ligand recognition within the eLP2 of the EP3 receptor have been reported as an important determinant of ligand selectivity (19). These results suggested that the extracellular domains of other prostanoid receptors are involved in the initial specific ligand interaction. The residues identified from the NMR approach for the interaction between the ligand and the eLP2 were further confirmed by site-directed mutagenesis. Results from the studies provided an approach of using NMR experiments guided site-directed mutagenesis for identification of the important residues of other prostanoid receptors and other G-protein-coupled receptors, which is more reasonable and close to the fact than those mutations performed only based on alignment.

Thromboxane A2 (TXA2) is a potent platelet aggregatory and vasoconstrictive mediator (2). The function of TXA2 is mediated by specific cell surface receptor, thromboxane A2 receptor (TP) (3). The understanding of the structure and function of TP receptor can greatly explain how the ligand binds to its receptor and initiates the following cell signaling.

TP receptor was first purified from platelet in 1989, and the cDNA of TP receptor was cloned from placenta in (4, 5). Other human prostanoid receptor cDNAs have also been cloned by homology screening. All of the prostanoid receptors belong to the G-protein-coupled receptor family that share a basic seven transmembrane segments and couple to different signal transduction systems to play diverse physiological and pathological roles (6–14). TXA2 binds to TP receptor and triggers an increase of intracellular calcium. There were two TP receptor isoforms with different C-terminal tails, resulting from alternative splicing that the last 15 amino acids of the C terminus were replaced by 79 amino acids (15, 16). The two TP receptor isoforms coupled to the same signal transduction, but endothelium expressed only the spliced form and placenta expressed both types of the TP receptors (15–17).

Based on the sequence alignment, the second extracellular loop (eLP2) and the third and seventh transmembrane domains of the prostanoid receptors are highly conserved and are proposed to be involved in ligand binding (18). Residues 198–205 in the eLP2 of the EP3 receptor have been reported as an essential determinant of ligand selectivity (19). These results suggest that the extracellular domains of other prostanoid receptors are involved in the initial specific ligand interaction. The residues responsible for specific ligand recognition within the eLP2 of the TP receptor have not been thoroughly examined. The mutations based on alignment only are controversial and will need structural information to support. The structures of the transmembrane domains of prostanoid receptors may be similar, but the specific recognition sites on extracellular domains will be different because the ligand structures are different. Thus, structural characterization of the extracellular functional domains of prostanoid receptors could help in understanding the specificities of ligand binding. In our current study, the structure of the highly conserved eLP2 has been characterized by high resolution NMR using a synthetic eLP2 peptide with constrained loop ends (1). To identify which residues make up the ligand recognition site of the receptor, SQ29,548 was added to the peptide to determine the interaction using high resolution two-dimensional 1H NMR technique. The residues identified from the NMR approach for the interaction between the ligand and the eLP2 were further confirmed by site-directed mutagenesis. Results from the studies provided an approach of using NMR experiments guided site-directed mutagenesis for identification of the important residues of other prostanoid receptors and other G-protein-coupled receptors, which is more reasonable and close to the fact than those mutations performed only based on alignment.

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1 The abbreviations used are: TXA2, thromboxane A2; TP, thromboxane A2 receptor; NOESY, nuclear Overhauser effect spectroscopy; TOCSY, total correlation spectroscopy; HPLC, high performance liquid chromatography; DQF-COSY, double-quantum filtered correlation spectroscopy.
residues: Homo-Cys1, Arg2, Tyr2, Thr4, Val5, Gln6, Ser7, Tyr9, Pro10, Gly11, Ser10, Trp11, Cys12, Phe13, Leu14, Thr15, Leu16, Gly17, Ala18, Ser19, Gly20, Gly21, Asp22, Homo-Cys23. The proton chemical shifts for SQ29,548 with and without the TPeLP2 peptide are shown in Table 1.

**EXPERIMENTAL PROCEDURES**

**Materials**—Ethanol-d6, and D2O were purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA). SQ29,548 was from Cayman Chemical (Ann Arbor, MI). The QHaprep spin miniprep kit was from Qiagen (Valencia, CA). COS-7 cell was purchased from ATCC (Manassas, VA). Medium for culturing COS-7 cells was from Invitrogen (Carlsbad, CA). Medium for culturing COS-7 cells was from Invitrogen. Materials for culturing COS-7 cells was from Invitrogen.

**Peptide Synthesis**—A constrained loop peptide mimicking the sequence of the second extracellular loop of TP receptor (residues 173–180) with homocysteine added at both ends (Fig. 1) was synthesized for NMR study using fluorenylmethoxycarbonyl-polyamide solid phase method and cyclized by the formation of disulfide bonds as described previously (1, 20–22). Briefly, the peptide was purified to homogeneity by HPLC. For the cyclization, the peptide was dissolved in 1 ml of dimethyl sulfoxide (Me2SO) and added to H2O at a final concentration of 0.02 mg/ml with pH 8.5 adjusted by triethylamine, and stirred overnight at room temperature. The cyclic peptide was then lyophilized and purified by HPLC on the C4 column.

**NMR Sample Preparation**—The HPLC-purified constrained loop peptide was dissolved in 20 mM sodium phosphate buffer, pH 6.0, at a final concentration of 5 mM. 1 mg of SQ29,548 was dissolved in 50 μl of ethanol-d6 and then added to 0.45 ml of sodium phosphate buffer (20 mM) containing 10% D2O (11). Any insoluble ligand was removed by centrifugation. The concentration for the mixture of peptide and SQ29,548 was the same as above.

**NMR Experiments**—Proton NMR experiments were carried out on a VARIAN Unity Plus 500 spectrometer, which was equipped with 1H- and 13C-detected 2D-EXSY, 2D-TOCSY, 2D-NOESY and 2D-HEROES experiments. All the NMR spectra were collected using standard procedures. The NMR experiments were performed in the presence of scalar interactions spin-lock sequence with a total mixing time of 50 ms.

**RESULTS**

**NMR Study of TP eLP2 Interacted with SQ29,548**—The constrained synthetic eLP2 peptide (Fig. 1) mimicking the second extracellular loop of the native human TP receptor, which showed the conformational change upon the interaction with the receptor antagonist SQ29,548 in the circular dichroism (CD) and fluorescent spectroscopic studies (1), was...
used for the two-dimensional $^1$H NMR experiments. To observe the interaction between the constrained peptide and SQ29,548 at the atomic level, NOESY spectra for the eLP2 peptide, SQ29,548, and the mixture of the peptide with SQ29,548 were recorded separately under the same conditions as described under “Experimental Procedures.” Resonance assignments were made using a standard approach (Table I) (26). To determine which residues of the constrained eLP2 interact with the ligand SQ29,548, the NOESY spectra were used to identify the intermolecular contact between the peptide and SQ29,548 (Fig. 2). The results indicate that Val176, Leu 185, Thr 186, and Leu 187 interacted with SQ29,548 and predict that the residues are involved in the TP receptor initial ligand recognition.

![Fig. 1. Topology model of the TP receptor.](image)

![Fig. 2. Expanded region of TOCSY spectrum of SQ29,548 (A), NOESY spectra of TP eLP2 peptide (B), and the mixture of TP eLP2 peptide and SQ29,548 (C).](image)

![Fig. 3. A, Western blot of the recombinant human TP receptors expressed on COS-7 cells. 50 μg of COS-7 cells transfected with wild-type (TPwt) or mutant TP receptor cDNA were subjected to SDS-PAGE and transferred onto a nitrocellulose membrane. The membrane was probed with rabbit anti-TP peptide antibody. The line on the left side showed the position of TP receptor protein. B, the ligand binding activities of wild-type and mutant TP receptors. The $[^3]$H]SQ29,548 binding was assayed for COS-7 cell transfected with wild-type or mutant TP receptors. Unlabeled (cold) SQ29,548 was added as a competitive ligand for the assay. The binding activity of wild-type receptor was considered as 100%.](image)

![Fig. 4. The amino acid sequence of wild-type and the type of mutated residues (bold) of the recombinant TP receptors are indicated.](image)
Recombinant TP Receptors—To test whether the residues Val^{176}, Leu^{185}, Thr^{186}, and Leu^{187} identified by the NMR experiments using the constrained eLP2 peptide are involved in the ligand recognition for the native TP receptor, a series of recombinant protein of the human TP receptor with point mutation at the four residues were constructed. These four residues Val^{176}, Leu^{185}, Thr^{186}, and Leu^{187} were first replaced with glycine to eliminate the side chains of the residues. After transfection of the cDNA of the recombinant TP receptors into COS-7 cells, the similar expression level of the TP receptors were confirmed by Western blot (Fig. 3A). The binding of the recombinant receptors to its ligand was then performed using \[^{3}H\]SQ29,548, and unlabeled (cold) SQ29,548 was used as a competitive ligand (Fig. 3B). All of the mutants with glycine replacement showed decreased or lost binding activity to the receptor antagonist, SQ29,548, as compared with the TP wild type (Fig. 3B). These data indicate that the side chains of the residues Val^{176}, Leu^{185}, Thr^{186}, and Leu^{187} of the native TP receptor are important to the ligand binding via a direct contact in the ligand-binding site or an indirect induced structural effect. These results also support the conclusion based on the NMR experiments in which the four residues of the TP receptor play important roles on the receptor ligand interaction.

To further identify what determines the ligand binding, the four residues were then mutated to either the same type residues, residues with different structures, or residues with different charged (Fig. 4). Val^{176} was mutuated to residue Asp, Leu, or Arg, Leu^{185} and Leu^{187} were mutated to residue Ala, Asp, or Arg, Thr^{186} was mutated to residue Ala, Arg, or Ser. The cDNAs of the mutated receptors were obtained using standard PCR approach and then transfected into COS-7 cells. The expression of the recombinant TP receptors were confirmed by Western blot (Fig. 5A). The binding of the mutated TP receptors to SQ29,548 was shown in Fig. 5. Only one recombinant TP receptor with a V176L mutation retained the binding activity to SQ29,548 (Fig. 5B). All other mutants showed significantly decreased or lost binding activity (Fig. 5, B–E). In contrast, the control mutants of the TP receptor, Y178W and S181T, which are highly conserved in all the prostanoid receptors (Fig. 6), remained full binding activities to SQ29,548 as compared with the wild-type TP receptor (Fig. 7). These results indicate that the hydrophobic side chain of Val^{176} is important for the interaction with SQ29,548. For the residues Leu^{185}, Thr^{186}, and Leu^{187}, any structural changes to the side chain will affect TP receptor binding to its antagonist.
As compared with the wild-type TP ligand, SQ29,548, the receptor, Y178W and S181T retained all the binding activity to its ligand, SQ29,548. Previous studies from other groups also provided evidence to support the hypothesis that the second extracellular loop of the TP receptor is involved in the receptor ligand recognition. These studies supported the separated CD spectroscopic studies (1). The interaction was also supported by the separated fluorescence spectroscopic studies (1). The combination of the two-dimensional NMR experiments and the NMR experiment-guided mutagenesis approach provided a quicker way to identify the important ligand recognition site of the TP receptor. This approach can be used to characterize the ligand binding to other domains of the receptor.

During our preparation of this manuscript, Le Breton's group reported a mapping of the ligand-binding site of the human TP receptor using photoaffinity labeling and site-specific antibody probes (28). The antibody screening revealed that inhibition of the amino acid region Cys183-Asp185 was critical for radioligand binding and platelet aggregation. The studies provided evidences that the ligand interacts with amino acids within the second extracellular loop of the TP receptor (28). It further supported our conclusion described in this paper in which the four residues Val176, Leu185, Thr186, and Leu187 within the second extracellular loop are identified as important residues for the receptor ligand recognition. In comparison, the combination of the two-dimensional NMR experiments and the NMR experiment-guided mutagenesis approach could give detailed structural information about the interaction of the receptor and ligand, which could not be achieved by other approaches, including general mutation approach, photoaffinity labeling, and site-specific antibody screening. The agreement among our conclusion with the photoaffinity labeling and site-specific antibody investigation has further supported the reliability of the NMR experiment-based mutagenesis approach used for the identification of the ligand recognition site of the TP receptor. One of the key factors in these studies is to design a synthetic peptide with biological function. By using a constrained peptide to mimic the extramembrane loops of TP receptor, we successfully identified the ligand recognition site for the receptor.

Our identification of the important residues of TP eLP2 responsible for the contact with TP receptor ligand reported here does not exclude the other possible ligand-binding sites reported by other groups. We suspected that the ligand-docking site might differ from the final ligand-binding site, because the residues important to TP receptor ligand binding located within the transmembrane domains are conserved. The initial docking residues of the prostanoid receptors with their ligand shall be specific. The traditional alignment-based mutagenesis
approach may pick up some residues, which may not be involved in direct ligand contact, but which indirectly affect the protein activity through the change of protein conformation distantly. Nevertheless, our proton level information for identification of the TP receptor ligand recognition site on the extracellular domain will serve as a very valuable tool to characterize the structure of the TP receptor ligand docking site and understand the biological mechanism of TXA$_2$ binding to its receptor. In addition, it also provides great reference information to determine the ligand-docking sites for other prostanoid receptors, and understand the specific recognition among the eight different prostanoid receptors. In general, the NMR experiment-based mutagenesis approach is also suitable for identification of the ligand recognition sites for other G-protein-coupled receptors.

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