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Assembling NMR Structures for the Intracellular Loops of the Human Thromboxane A₂ Receptor: Implication of the G Protein-Coupling Pocket†

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Abstract

It has been reported that the multiple intracellular loops (iLPs) of the thromboxane A₂ receptor (TP) are involved in the receptor G protein coupling. In this study, a high-resolution 2D NMR technique was used to determine the 3D structures of the first, second, and third iLPs of the TP receptor using synthetic peptides constrained into the loop structures. 2D ¹H NMR spectra, TOCSY and NOESY were obtained for the two peptides from proton NMR experiments. The NMR data was processed and assigned through the Felix 2000 program. Standard methods were used to acquire sequence-specific assignments. Structure calculations were processed through DGII and NMR refinement programs within the Insight II program. We were able to calculate and use the NOE constraints to obtain the superimposed structure of ten structures for each iLP peptide. The NMR-determined structures of the iLP peptides were used to refine a homology model of the TP receptor. A 3D G-protein binding cavity, formed by the three intracellular loops, was predicted by the docking of the C-terminal domain of the Gαq. Based on the structural model and the previous mutagenesis, the residues in the TP intracellular loops (R130, R60, C223, F138) and in the Gαq (L360, V361, E358, Y359), which are important for interaction of TP with the G protein, were further highlighted. These results reveal the possibly important molecular mechanisms in TP receptor signaling and provide structural information to characterize other prostanoid receptor signalings.

INTRODUCTION

Prostanoids, a family of oxygenated, polyunsaturated fatty acids formed via the cyclooxygenase (COX) pathway from arachidonic acid, consist of prostaglandins, prostacyclins, and thromboxanes. They behave like local hormones that act in the vicinity of their production sites [1-2]. Each prostanoid exerts their function by binding to its specific prostanoid receptor. All known prostanoid receptors belong to the rhodopsin-type G protein-coupled receptor (GPCR) superfamily [3-11]. G protein-coupled receptors (GPCRs) form a large superfamily of membrane proteins with seven transmembrane domains [12]. Prostanoid receptors bind a ligand molecule on the extracellular side of the membrane and then interact with heterotrimeric G proteins on the intracellular side [3-11]. Recombinant protein studies have implied that the

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N- and C-terminal portions of the third intracellular loop (iLP3), and the cytoplasmic tail of the receptors are involved in the G protein activation and signal transduction. Other studies have shown that in the second intracellular loop (iLP2), the membrane-proximal portions of the iLP3, and the N-terminal segment of the cytoplasmic tail both contain amino acids which have been predicted to play a role in regulating the selectivity of G protein and GPCR interactions [13-23].

Understanding the specific details of the coupling between prostanoid receptors and their specific G proteins is dependent upon the structural characterization of the intracellular loop (iLP) domains of the receptor and the binding site of the G protein. However, the lack of a 3D structure for the receptor has become a major obstacle in understanding the molecular mechanisms regarding the detailed interaction between the receptor and G protein. Prior to this experiment, the 3D structure of the three extracellular loops and the first intracellular loop of the TP receptor had been configured by a high-resolution 2D NMR technique. Our focus in this study is the structural characterization of the second and third intracellular loops of the TP receptor. We hypothesize that the structures of the iLP2 and iLP3 regions are crucial to the determination of the specific binding sites where the G protein comes in contact with the TP receptor.

EXPERIMENTAL PROCEDURES

Materials

D₂O and dodecylphosphocholine-d38 (DPC-d38) were purchased from Cambridge Isotope Laboratories (Andover, MA). Trifluoroacetic acid (TFA) was obtained from Millipore (Bedford, MA). All other chemicals were from Sigma.

Peptide Synthesis

Using the fluorenylmethoxycarbonyl-polyamide solid phase method, we were able to synthesize the peptides mimicking the TP receptor's iLP2 (residues 129–149) and iLP3 (residues 220–246) by adding homocysteine (hCys) at both ends of each loop [24]. The peptide was purified to homogeneity by HPLC in 0.1% TFA. For the cyclization of the peptide (by disulfide bond formation), the purified peptide was dissolved in 1 ml DMSO and then diluted to a final concentration of 0.02 mg/ml. The solution was adjusted to pH 8.5 and then stirred overnight at room temperature. Next, the cyclic peptide was lyophilized and purified by HPLC using a C4 column. Molecular weights of the peptides were determined by mass spectrometry. The sequences of the synthesized TP iLP2 and iLP3 peptides are shown in Figure 1.

NMR experiments

NMR experiments were performed on a BRUKER AVANCE 600 MHz NMR instrument. All 2D experiments (TOCSY and NOESY) were performed at 298K, in 20mM sodium phosphate buffer (pH 6.0) containing 10% H₂O for the purpose of providing a lock signal. NOESY spectrum was recorded with a mixing time of 200ms; TOCSY spectrum was carried out with MLEV spin-lock sequence with total mixing time of 80 ms. The WATERGATE method [25] was used to suppress the solvent signal. Chemical shift was measured in reference to the remaining water peak, which is set at 4.7 ppm. All spectra were composed of 2048 complex points in the F2 and 512 complex points in the F1 with 64 scans per t1 increment. Quadrature detection was achieved in F1 by the States-TPPI method. The NMR data were processed using the Felix 2000 program (Accelrys Inc, San Diego). Sinbell window functions with either 0° (for DQF-COSY), 70° (for TOCSY), or 90° (for NOESY) shift were used in both dimensions. The sequence-specific assignment was obtained using a standard method [26].

Calculation of Structures

Felix 2000 software was used to convert the NOE cross-peak volumes into distance constraints. The distance-bound method was used during this procedure. NOE cross-peaks volumes were acquired and classified using a statistical classification function and characterized as strong, medium, or weak NOEs, in correspondence to the upper bound distances of 2.7, 3.5, and 5.0 Å [26,27]. The lower bound distance was set to 1.8 Å. Dihedral angle constraints were derived from the $^3J_{\text{NH}\alpha}$ coupling constants measured from the DQF-COSY spectrum. Distance geometry calculations were carried out on an SGI workstation using the DGII program within the Insight II package (Accelrys Inc, San Diego). Energy refinement calculations, such as restrained minimization or dynamics, were carried out in the best distance geometry structures using the Discover program within the Insight II package [26,27].

Molecular Modeling of the TP receptor and refinement using the NMR-determined iLP structure

Homology modeling within the Insight II program was used to build the structural working model for the TP receptor based on the bovine rhodopsin crystallographic structure [28]. The sequence of the human TP receptor was aligned with the bovine rhodopsin sequence. The model of the human TP receptor was first constructed with seven transmembrane (TM) helices by copying the coordinates of structurally conserved TM domains directly from the bovine rhodopsin protein. The loops that connect the TMs in the initial model were created by searching the database of known protein structures. After the structure of the TP iLP peptides were determined by NMR, these experimentally determined structures were connected to the TM domains and this structure model was further refined by energy minimization with fixed constraints on the TM domains.

RESULTS

Design and synthesis of the constrained peptides mimicking the TP iLP2 and TP iLP3 domains

It is believed that the seven TM domains of the GPCRs have adopted a similar conformation. Based on the TM domain model of the human TP receptor that was previously created by homology modeling using the X-ray structure of bovine rhodopsin as a template [29-32], the distance between the iLP2 termini, connected to the third and fourth TM domains is about 12.9 Å and the distance between the iLP3 termini connected to the fifth and sixth TM domains is about 12.4 Å (Figure 1A). Two peptides corresponding to the TP iLP2 and TP iLP3 regions (Figure 1B), which contained additional homocysteine residues at the N- and C-termini, were synthesized and constrained to a similar distance at the termini by a disulfide bond formation using a the fluorenyl-methoxy-carbonyl-polyamide solid phase method (Figure 1C) [27, 29-36]. After synthesis, the peptides were purified to homogeneity by reverse phase HPLC using a C4 column, and the correct molecular weights were confirmed by mass spectrometry analysis [37].

NMR experiments and resonance assignments

To characterize the structural features of the constrained peptides mimicking the TP iLP2 and TP iLP3 domains, high-resolution ^1H 2D NMR spectroscopy was used to determine their solution conformation in respect to the corresponding TM domains. ^1H 2D TOCSY, NOESY and DQF-COSY spectra were used to measure the constrained peptides. ^1H NMR assignments were accomplished using the standard sequential assignment technique described [26,27, 29-36]. The assignment procedures involved in the identification of spin systems using the TOCSY spectra, and the sequential assignments using NOESY spectra were completed by manual assignment. In addition, each step of the assignments was further verified by using an

auto-assignment program following the instructions in the Felix 2000 program package. Using the 2D homonuclear sequential assignment procedure, we were able to get nearly complete proton resonance assignments for the TP iLP2 and TP iLP3 peptides. The chemical shift of the protons in TP iLP2 and TP iLP3 are summarized in Table 1 and 2, respectively. After the resonance assignments were achieved, distance constraints for the intra- and inter-residue protons were generated from the NOESY cross-peaks using the Felix 2000 NMR assignment package. As many as 310 constraints (92 intraresidue, 127 sequential, and 91 long range) were obtained for the TP iLP2 (Figure 2A), whereas a total of 195 constraints (96 intraresidue, 61 sequential, and 38 long range) were obtained for TP iLP3 (Figure 2B). The numbers of the constraints per residue were plotted in Figure 2. In addition, dihedral angles were obtained from the DQF-COSY spectra for the corresponding peptides.

Solution structures of the constrained TP iLP2 and TP iLP3 domains

The distance constraints and dihedral angle constraints from the assigned NMR spectra were input into the NMR refinement module of the Insight II 2000 program package to calculate the initial 3D structures of the TP iLP2 and TP iLP3 domains using the DGII calculation program (Figures 3A and C), and then using the energy refinement calculation program (Figures 3B and D). The structure of the TP iLP2 peptide shows a large turn structure formed by several bend conformations within residues E2-G6, I7-S12 and V16-Q19 (Figure 3B). The structure of the TP iLP3 peptide shows a helical-like conformation in both the N- and C-terminal regions with a turn structure located at residues 15–18 (Figure 3D). These 3D structural results are also consistent with the secondary structure analyses from the H α Chemical Shift Index (Figure 4).

Linking of the three individual NMR TP iLP structures to the corresponding TM domains in the TP model

The NMR structures of the three individual constrained TP iLP segments were refined by energy minimization and dynamic studies. The hCys residues at the peptide ends were deleted to form open loop conformations (Figures 5A and C). The N- and C-termini of the NMR structures of the previously determined iLP1 (data not shown, [31]) and the proposed iLP2 (Figures 5A and B) and iLP3 (Figures 5C and D) were connected to the corresponding terminal residues of the TM domains (Figures 5B and D) using the following five steps: 1) The open termini of the individual TP iLP segments were placed in the intracellular region with positions favoring bond formation with the corresponding terminal residues of the TM domains of the TP model using the Insight II program; 2) The common trans conformation for the peptide bond and the dihedral angles extending from the TM helix were used as a factor for determining the conjunction angles for all of the Loop-TM helix connections; 3) The small distance gaps between the open loop and the open TM helix were annealed together by rotating the terminal residues at both ends of the individual TP iLPs; 4) Energy statements were used to remove the violations between the connections and also to form a stable conformation for the links between the loops and TM helix and 5) The best conformation for the iLP-TM connection was determined by a 2000-step energy minimization calculation and dynamic studies using the Insight II program. The conformations of the TM-connected iLP domains (Figures 5B and D) have changed very little from the original structures (Figures 5A and C), indicating that the configuration of the iLP domains and the TM model did not cause structural perturbation for the determined NMR structures of the iLP domains.

Putative G protein-binding pocket formed by the three intracellular loops

The assembled structural model for the intracellular loops of the TP receptor provides an initial 3D structural image that lets us examine the relationship of the intracellular domains involved in forming a G protein-binding pocket (Figure 6). After analyzing the structural features of the iLPs, we were able to obtain very interesting information which was useful in further

identifying a putative G protein-binding pocket. First, the three iLPs interact together to form a globular conformation (Figure 6). Then a putative G protein-binding site is clearly shown by an arrangement, in which the iLP1 (Figures 6A and B (red)) is located above the iLP3 (Figures 6A and B (blue)), which forms a bed, and the iLP2 (Figures 6A and B (yellow)) is set towards the back of the proposed pocket (Figure 6C). This pocket, which is formed by the three loops, is consistent with the previous reports suggesting that more than one intracellular loop are involved in the contact with the G protein through mutagenesis studies [13,15-16,18-19,38]. The putative pocket is further supported by the presence of the key residues, which are important to the G protein coupling identified by mutagenesis, including R60 in the iLP1 [31], R130 and F138 in the iLP2 [38,20], and C223 in the iLP3. Figure 6 shows the protein structural folding without (Figure 6A) and with the side chains (Figure 6B) as well as the protein surface display (Figure 6C). The four residues are surrounding the pocket and located just on the surface, which makes them available and likely to interact with the residues from the G protein. This is the first to predict the G protein-binding site for the prostanoid receptors based on the combined data obtained from mutagenesis and peptide NMR studies.

Docking of the receptor binding domain of the Gαq to the putative G protein-binding pocket

The C-terminal domain of the Gαq and Gαs that binds to prostanoid receptors has been identified by MINIGENE techniques [33]. Recently, the NMR 3D structure of the C-terminal domain (eleven residues) of Gαs, bound to a similar prostanoid receptor (the prostacyclin receptor) was solved by way of our NMR spectroscopy, and the key residues for the binding were narrowed down to four by a mutagenesis approach [33]. The information was used to model the 3D structure of the C-terminal domain of the Gαq (Figure 7A) and to dock the four residues with the putative G protein-binding pocket of the TP using the Insight II docking program. The four residues in the Gαq C-terminal domain could be fixed into the pocket using two possible orientations. Figures 7A and B demonstrate the first orientation in stick render mode and potential energy surface mode, respectively. Similarly, the second orientation is illustrated in Figures 7C and D. In the first orientation, the negative charge of the C-terminal carboxy group formed a charge contact with R60 and R130 (both with positive charges), while the L360 formed a hydrophobic contact with the side chain of F138 (Figure 7A). With these two contacts, the docking was achieved under a very favorable energy status (Figure 7A). The second orientation occurred when the E358 (also negatively charged) formed a charge contact with R60 and R130, while the L360 and V361 formed a hydrophobic contact with F138 (Figure 7C). It should be noted that the corresponding residues, E358, L360, and V361 of Gαq in the Gαs, which are important to the receptor binding, have been confirmed by mutagenesis [33]. Both of the docking models have suggested several additional residues in the three iLPs, which might be involved in the G protein binding (Figure 8).

DISCUSSION

Because the TP receptor is involved in mediating hypertension, stroke, thrombosis and other vascular diseases, it has become one of the most important prostanoid receptors targeted in the development of a new drug [39-40]. Since the receptor's cloning in 1991, more than one and a half decades have passed and still little structural information is available to help understand the receptor action mechanisms through specific G protein coupling. The reason for this is that there are only non-experimental protein structures available for the intracellular loops of the prostanoid receptor due to the difficulty in crystallizing the GPCRs. Even the homology modeling approach has not worked for the intracellular domains of the receptors due to the amino acid residue variations between the prostanoid receptor and the crystallized rhodopsin. Thus, finding an experimental approach for predicting the intracellular structures of the receptor could be a very valuable discovery.

Recently, the structure of the R60 residue located in the turn position of the human TP iLP1 was characterized by our NMR studies, and its importance in the G protein coupling, through charge contact with Gαq, has been identified by site-directed mutagenesis using Lys and Leu replacements [31]. This information has advanced our knowledge in the G protein interaction for the receptor. However the lack of the other two TP iLP structures would not allow us to provide better models for predicting the possible pocket of the G protein binding.

During the characterization of the structure and function relationship of the prostanoid receptors we have successfully developed a constrained peptide synthesis approach for mimicking the loop structures of proteins, including the TP receptor extracellular loops. The predicted ligand recognition site by the configured NMR structures of the synthetic extracellular loops of the TP receptor has been confirmed by a mutagenesis approach [32]. A similar approach has also been successfully used to determine the structure of the TP iLP1 [31]. This paper has described the NMR structural determination of the synthetic constrained peptides mimicking the iLP2 and iLP3 and explored the prediction of the binding pocket of the G protein based on the three intracellular loop configurations using the constrained peptide structures.

The NMR refined 3D model of the TP receptor has given us an important allusion to the G protein's coupling site on the intracellular loops of the receptor. Two different orientations and color schemes of the intracellular domain of the NMR refined structure model are shown in Figure 7. It shows a plier-like structure, formed predominantly by residues on the iLP2 and iLP3 (Figure 8). The model is further validated by demonstrating that the G protein-binding pocket is surrounded by the residues, R60, R130, F138 and C223, which have been identified as key residues involved in G protein coupling by site-directed mutageneses (Figure 6). Based on the NMR model (Figure 7), it would be valid to suggest that the positively charged residues, Arg60 in the iLP1 and Arg130 in the iLP2 could potentially have electrostatic interaction with the G protein, while the F138 in the iLP2 could possibly serve as a hydrophobic residue in contact with the G protein. The additional surface residues involved in the G protein coupling formed by the three intracellular loops can be predicted based on the following: The NMR refined structure model indicated that the residues forming the plier-like structure consisted of the additional residues, R140, R136, V143, S145, and R147 on the iLP2, as well as E230 and A232 on the iLP3 (Figure 8). There are many ongoing site-directed mutagenesis studies focused on identifying the functions of those residues in the receptor signaling. Finally, the model also suggested that the role of the previously identified important residue, R60, may also be to possibly interact with the E129, which may stabilize the structure of the intracellular domain (data not shown).

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Abbreviations

TP, thromboxane A₂ receptor
G protein, guanine nucleotide binding protein
GPCR, G Protein-Coupling Receptor
TM, transmembrane domain
iLP, intracellular loop
NMR, nuclear magnetic resonance
DQF-COSY, double-quantum filtered correlation spectroscopy
TOCSY, total correlation spectroscopy

NOESY, nuclear Overhauser effect spectroscopy
HPLC, high performance liquid chromatography
hCys, homocysteine.

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Figure 1.

(A) The seven TM domain (TM1-TM7) model of the human TP receptor. This 3D structure was constructed using homology modeling within the Insight II program. The distances between the iLP2 termini (connecting the third and fourth TM domains) and between the iLP3 termini (connecting the fifth and sixth TM domains) are shown. (B) Topology model of the TP receptor. The heavy lines represent the regions that were emphasized for these studies. (C) The peptide mimicking the human TP receptor iLP2 (residues 1–23) and iLP3 (residues 1–29), is shown with homocysteine added at both ends of the loop.

Figure 2. Number of constraints per residue for the TP iLP2 (A) and TP iLP3 (B) peptides derived from NMR structural analyses.

Figure 3.

NMR solution 3-D structures of the constrained TP iLP2 and TP iLP3 peptides.

Superimposition of ten structures using the α -carbons of TP iLP2 (A) and TP iLP3 (C) after DGII calculations based on the NOE constraints and dihedral angles. Superimposition of ten structures using the α -carbons of TP iLP2 (B) and TP iLP3 (D) obtained from the energy refinement calculations.

Figure 4. Secondary structure analyses by H α Chemical Shift Index for TP iLP1 (A) and TP iLP2 (B).

Figure 5.

The arrangement of TP iLP2 (A, B) and TP iLP3 (C, D) in respect to TM3 and TM4 (A, B), and TM5 and TM6 (C, D) of the TP receptor working model. The distance between TM3 and TM4 is 12.99 Å (A) while the distance between TM5 and TM6 is 12.41 Å (C). The distances between the termini of the NMR structure of iLP2 and iLP3 are 9.14 Å and 9.59 Å, respectively (A, C). The NMR structure of the iLP2 was connected between the TM3 and TM4, and iLP3 structure was connected between TM5 and TM6 of the TP receptor working model and an energy minimization was performed for the connected molecule. The resultant distance between TM3 and TM4 changed to 10.95 Å (B) while for TM5 and TM6 it changed to 14.96 Å (D). The distance between the termini of the NMR structure of iLP2 and iLP3 also changed to 13.31 Å and 15.96 Å, respectively.

Figure 6.

Structure model of the TP intracellular domain that was generated based on the NMR study, and a putative G protein-binding pocket, shown in three different schemes: (A) α -carbons only, (B) CPK mode with all the side chain and (C) potential energy surface. Some key residues, known to be important to the G protein-coupling from the mutagenesis studies are also shown.

Figure 7. Docking of the C-terminal domain of Gαq into the putative binding site on the TP intracellular domain. The first orientation is shown in stick render form (A) and potential energy surface mode (B). The second orientation is also shown in stick render form (C) and potential energy surface mode (D).

Figure 8.
The docking model is shown in potential energy surface mode. Some additional residues that might also be important for G protein coupling are indicated.

Table 1

Proton chemical shifts for iLP3 peptide

Residues	HN	Ha	H β	Others
hCys1	-	4.171	1.864, 2.605	2.444
Ala2	7.987	4.007	1.111	
Thr3	8.188	4.047	3.905	0.936
Leu4	8.082	4.133	1.277, 1.344	0.587, 0.643, 1.291
Cys5	7.951	4.222	2.866	
His6	7.783	4.373	2.800, 2.924	6.672, 7.360
Val7	7.766	3.761	1.626	0.475, 0.556
Tyr8	8.192	4.27	2.644, 2.655	6.500, 6.825
His9	8.285	4.375	2.783, 2.920	6.871, 7.996
Gly10	7.753	3.624, 3.677	-	-
Gln11	8.23	4.035	1.726, 1.817	2.095, 7.356
Glu12	8.417	3.962	1.712, 1.785	2.023
Ala13	8.164	3.996	1.102	-
Ala14	8.118	3.955	1.103	-
Gln15	8.047	4.03	1.722, 1.854	2.098, 2.140, 7.356
Gln16	8.27	4.074	1.722, 1.849	2.094, 7.385
Arg17	8.236	4.348	1.463, 1.575	1.407, 2.928, 7.047
Pro18	-	4.152	1.653, 2.048	1.756, 3.370, 3.542
Arg19	8.384	4.01	1.504, 1.598	1.409, 2.928, 7.047
Asp20	8.255	4.309	2.388, 2.472	-
Ser21	8.081	4.128	3.604, 3.681	-
Glu22	8.117	3.958	1.808	2.02
Val23	7.808	3.719	1.809	0.670, 0.701
Glu24	8.163	3.965	1.803	2.02
Met25	-	-	-	-
Met26	8.232	4.13	1.733	1.983
Ala27	8.04	3.984	1.111	-
Gln28	8.167	4.039	1.722	2.098, 7.410
hCys29	8.368	4.069	-	-

Table 2

Proton chemical shifts for iLP2 peptide

Residues	HN	H α	H β	Others
hCys1	-	-	-	-
Glu2	8.665	4.357	1.877, 1.945	2.347
Arg3	8.136	4.239	1.720, 1.808	1.622, 3.132, 7.136, 8.097
Tyr4	8.116	4.524	2.896, 2.936	6.729, 7.038
Leu5	8.033	4.259	1.485, 1.509	0.759, 0.823
Gly6	7.621	3.808	-	-
Ile7	7.753	4.190	1.828	0.848, 1.093, 1.387
Thr8	8.048	4.278	4.112	1.112
Arg9	8.371	4.229	1.514, 1.661	1.436, 3.097, 7.092, 8.302
Pro10	-	4.352	1.818, 1.975	2.225, 3.583, 3.734
Phe11	7.930	4.494	3.024, 3.112	7.185, 7.244, 7.278
Ser12	7.959	4.278	3.808, 3.891	-
Arg13	8.356	4.229	1.508, 1.642	1.436, 3.083, 7.097, 8.293
Pro14	-	4.244	1.867, 2.068	1.691, 3.475, 3.646
Ala15	8.230	4.146	1.357	-
Val16	7.896	3.994	2.024	0.887
Ala17	8.033	4.259	1.485	-
Ser18	8.033	4.323	3.788, 3.847	-
Gln19	8.155	4.263	1.945, 2.083	2.308, 8.033, 8.092
Arg20	8.146	4.543	1.710, 1.808	1.573, 3.102, 7.116, 8.092
Arg21	8.141	4.524	1.720, 1.808	1.612, 3.141, 7.151, 8.092
Ala22	8.008	4.200	1.348	-
hCys23	8.062	4.264	1.955, 2.073	2.289