A Simple, Quick, and High-Yield Preparation of the Human Thromboxane A_2 Receptor in Full Size for Structural Studies[†]

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ABSTRACT: Human thromboxane A_2 receptor (TP), a G protein-coupled receptor (GPCR), is one of the most promising targets for developing the next generation of anti-thrombosis and hypertension drugs. However, obtaining a sufficient amount of the full-sized and active membrane protein has been the major obstacle for structural elucidation that reveals the molecular mechanisms of the receptor activation and drug designs. Here we report an approach for the simple, quick, and high-yield preparation of the purified and active full-sized TP in an amount suitable for structural studies. Glycosylated human TP was highly expressed in Sf-9 cells using an optimized baculovirus (BV) expression system. The active receptor was extracted and solubilized by different detergents for comparison and was finally purified to a nearly single band with a ratio of 1:0.9 ± 0.05 (ligand:receptor molecule) in ligand binding using a Ni column with a relatively low yield. However, a high-yield purification (milligram quantity) of the TP protein, from a modulate scale of transfected Sf-9 cell culture, has been achieved by quick and simple purification steps, which include DNA digestion, dodecyl-maltoside detergent extraction, centrifugation, and FPLC purification. The purity and quantity of the purified TP, using the high-yield approach, were suitable for protein structural studies as evidenced by SDS−PAGE, Western blot analyses, ligand binding assays, and a feasibility test using high-resolution one-dimensional and two-dimensional ^{1}H NMR spectroscopic analyses. These studies provide a basis for the high-yield expression and purification of the GPCR for the structural and functional characterization using biophysics approaches.

Because of its importance in mediating thrombosis (*1, 2*), hypertension (*3*), and cancers (*4*), the elucidation of thromboxane A_2 (TXA_2)[1] signaling through its receptor, the TXA_2 receptor (TP), is one of the most attractive studies in eicosanoid research. The human TP cDNA, which was first cloned from placenta in 1991, encodes a protein of 343 amino acid residues (*5*). The cDNA for another TP receptor was isolated from human endothelial cells, which has a different C-terminal tail, resulting from alternative splicing (*6*). These receptors are the same in regard to their signal transduction; however, the endothelium expressed only the spliced form, and the placenta expressed both types of the TP (*6, 7*). Like other prostanoid receptors, including the prostaglandin D_2 (*8*), E_2 (*9–14*), F_2 (*15*), and I_2 (*16*) receptors, TP belongs to the G protein-coupled receptor (GPCR) family and is composed of three intracellular loops (iLPs) and three extracellular loops (eLPs) connecting seven transmembrane (TM) helices. So far, no solution or crystal structures of any of the eight full-sized prostanoid receptors are available due to the lack of the large amount of purified receptors required (high-milligram range) for biophysics studies. The earlier baculovirus (BV) expression systems for TP (*17, 18*) were able to generate the receptor protein only within the microgram range, which was only sufficient for Western blot, binding assays (*19*), and mass spectrometric analysis (*20*).

Recently, through a combination of the NMR spectroscopy technique, site-directed mutagenesis, peptide mimicking, and molecular modeling (*21–26*), we have revealed the ligand recognition site in the extracellular domains and found that the second extracellular loop (*22, 25, 26*) plays a critical role in forming the ligand recognition pocket (*22, 24–26*). However, understanding the detailed molecular mechanisms for the ligand recognition, binding, and signaling activation of the native receptor remains critical. This will require studies of the structure−function relationship using a purified, active receptor at full size.

In this study, we focus on obtaining a large quantity of the full-sized human TP in a purified and active form, suitable for structural characterization using biophysics approaches, by establishing a simple, quick, and high-yielding expression and purification system. The studies have demonstrated that the purified human TP, in its full size and active form, could be produced at the high-milligram level from a modulate-

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[1] Abbreviations: TXA_2, thromboxane A_2; TP, TXA_2 receptor; NMR, nuclear magnetic resonance; TOCSY, total correlation spectroscopy; 1D, one-dimensional; 2D, two-dimensional: OG, *n*-octyl β-D-glucopyranoside (octyl glucoside); DM, *n*-dodecyl β-D-maltoside (dodecyl-maltoside); PCR, polymerase chain reaction; GPCR, G protein-coupled receptor; PBS, phosphate-buffered saline; PMSF, phenylmethanesulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis; U46619, 9,11-dideoxy-9α,11α-methanoepoxy prostaglandin $F_{2\alpha}$.

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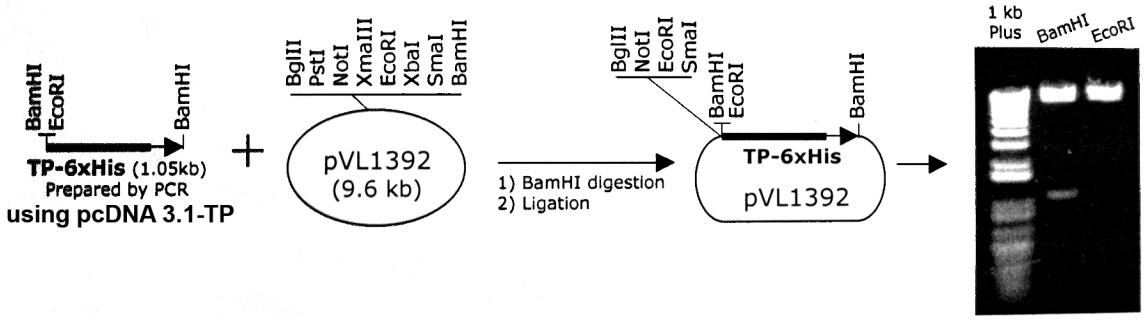

FIGURE 1: Subcloning of the human TP cDNA into the BV vector. The subcloning was generated by Norclone (London, Ontario, Canada).

scale of Sf-9 insect cell culture. The quality and quantity of the purified receptor were suitable for characterization of the receptor using high-resolution NMR spectroscopy and provided a basis for the elucidation of the more detailed structure−function relationship of the receptor, which shall be revealed in the near future.

EXPERIMENTAL PROCEDURES

Materials. D_2O and DSS (2,2-dimethyl-2-silapentane-5-sulfonic acid) were purchased from Cambridge Isotope Laboratories (Andover, MA). COS-7 and HEK293 cell lines were purchased from ATCC (Manassas, VA). The Sf-9 cell line and all the media for culturing the cells were purchased from Invitrogen (Carlsbad, CA). [^{3}H]SQ29,548 was purchased from Amersham Pharmacia Biotech (Piscataway, NJ). Unlabeled SQ29,548, and rabbit anti-human TP receptor antibody were purchased from Cayman Chemicals (Ann Arbor, MI). DNase was purchased from Sigma (St. Louis, MO).

Subcloning of the Full-Sized Human TP into the BV Vector. The cDNA of the human TP (343 residues) was isolated from a pcDNA3.1 vector, previously constructed and used to express the active TP receptor in the mammalian cells, HEK293 and COS-7 (*23, 25*). A six-His tag DNA sequence was linked to the isolated TP cDNA at the C-terminal position of the receptor protein to generate the pVL1392-TP-6His construct using a PCR approach. The correct insert and sequence of the TP-6His cDNA were confirmed by restriction enzyme cutting and DNA sequencing analysis (Figure 1).

Construction of the Recombinant BV for TP Expression. The construction of the recombinant BV for TP expression was conducted in the Baculovirus/Monoclonal Antibody Facility at the Baylor College of Medicine (Houston, TX), based on our experimental designs. Briefly, the cotransfection of the cultured Sf-9 insect cells was performed using the wild-type Autographa Californica nuclear polyhedrosis virus (AcNPV), the pVL1392-TP-6His plasmid, and the isolated recombinant BV that arose by homologous recombination. The packed, recombinant BV in the culture supernatant of the transfected cells was plaque-purified by an agarose overlay assay. Individual recombinant viral plaques were plucked from the agarose, extracted, and used to infect Sf-9 insect cells. The infected Sf-9 cells, using the different viral plaques, were subjected to identification of the initial expression of the receptor.

Optimization of the Expression of the Human TP Receptor. Ten viral isolates of the Sf-9 cells infected with 10 different viral plaques (A−J, Figure 2) were cultured in 5 mL of

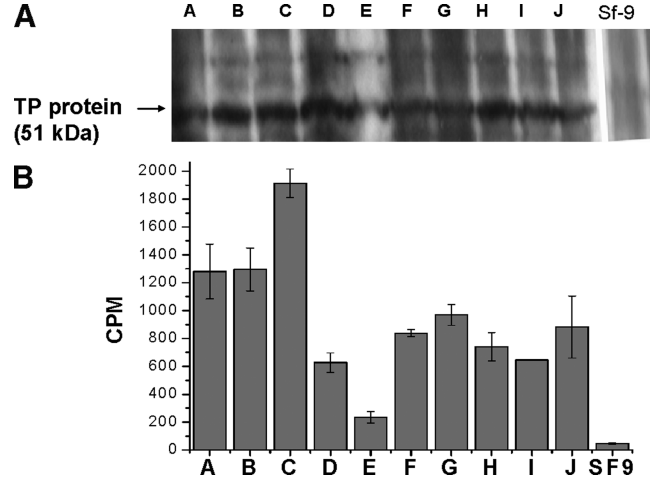

FIGURE 2: Screening of the TP expression in Sf-9 cells using different recombinant BV plaques. (A) Western blot analysis for the Sf-9 cells expressing TP. The Sf-9 cells (approximately 10 μg of protein/lane) infected with the BV-containing TP cDNA were subjected to SDS−PAGE and transferred onto a nitrocellulose membrane, which was probed with rabbit anti-TP peptide antibody. The position of the TP receptor protein is indicated at the left. (B) Binding of [^{3}H]SQ29,548 to the Sf-9 cells expressing the TP protein. The binding of the TP antagonist to the cells was assessed by incubating the cells with 3.1 nM [^{3}H]SQ29,548. The results are representative data from three assays (n = 3) and are shown as means ± the standard error.

Grace's insect medium. The cells were collected by centrifugation, and the expression of the TP was examined by ligand binding assays and Western blot analysis. One of the viral isolates was also used for determining the highest expression yield on different days following the infection. Under these conditions, an optimized expression system with the best viral plaque and optimized harvesting time was used for expression of the TP in large-scale production.

Electrophoresis and Western Blot. Different amounts of the TP protein (5−20 μg) were subjected to SDS−PAGE on a 10% polyacrylamide gel and then stained by Coomassie staining or transferred to a nitrocellulose membrane for Western blot, in which a band recognized by anti-human TP receptor antibody was visualized by a second antibody linked to horseradish peroxidase as described previously (*23*). For the deglycosylation experiment, TP was digested with peptide:*N*-glycosidase F (PNGase F) (New England Biolabs) according to the manufacturer's protocol. The TP was denatured with 5% SDS and 0.4 M DTT at 100 °C for 10 min. The reaction was initiated by adding 50 mM Na_3PO_4 buffer (pH 7.5), 1% NP-40, and 2 μL of PNGase F, and the mixture was incubated at 37 °C for 1 h.

Ligand Binding Assay. Ligand binding assays for the TP were performed using the methods described previously (*17-19, 25*). The protein sample was mixed with Tris-HCl buffer (25 mM, pH 7.4) containing 5 mM $CaCl_2$ and incubated with varying amounts of [^{3}H]SQ29,548 in a 0.1 mL reaction volume with vigorous shaking at room temperature for 60 min. The reaction was terminated by adding 1 mL of ice-cold washing buffer [25 mM Tris-HCl (pH 7.4)]. The unbound ligand was filtered through a Whatman GF/C glass filter (presoaked in ice-cold washing buffer) using a vacuum system. The radioactivity of the TP-bound [^{3}H]SQ29,548 remaining on the glass filter was counted in 4 mL of scintillation cocktail using a Beckman β counter. For the competitive inhibition assay, unlabeled SQ29,548 was added.

Affinity Purification (high purity with low yield) of the Active TP Protein from the Sf-9 Cells. A 300−500 mL suspension culture of Sf-9 cells expressing the TP was collected. The TP protein was extracted with 1% nonionic detergent, octyl glucoside (OG), containing 1 mM PMSF (phenylmethanesulfonyl fluoride) overnight at 4 °C. The extracted TP protein was separated by ultracentrifugation (250000*g* for 2 h at 4 °C). The supernatant was purified by the FPLC system using a Superdex-75 column (1 cm × 45 cm) at 4 °C. The fractions containing the active TP, according to the binding assays, were pooled and loaded onto a Ni column (His-Select Affinity Gel). After 2 h, the Ni column was washed using PBS with 0.5% OG and 10 mM imidazole to remove any unbound protein, and the bound TP was finally eluted with 250 mM imidazole in the same buffer. The purified TP was analyzed by binding assay, SDS−PAGE analysis, and Western blotting after the imidazole was removed by dialysis.

Simple, Quick, and High-Yield Purification of the Full-Sized and Active TP. A 500 mL suspension culture of the Sf-9 cells highly expressing the TP was washed twice with ice-cold PBS and collected by centrifugation. The Sf-9 cells were resuspended in PBS containing the low-critical micelle concentration detergent, *n*-dodecyl β-D-maltoside (DM) (1%), 1 mM PMSF, and 40 μg/mL DNase, and solubilized overnight at 4 °C on a rotating orbital shaker. The extracted TP protein was separated twice by ultracentrifugation (250000*g* for 2 h at 4 °C). The supernatant was purified by the FPLC system at 4 °C using a Superdex-75 column (1 cm × 45 cm) with a flow rate of 0.2 mL/min using PBS with 0.2 mM DM. The fractions containing the active TP protein were identified by binding assays, SDS−PAGE analysis, and Western blotting.

NMR Experiments. The purified TP (4 mg/0.5 mL) in 20 mM sodium phosphate buffer (pH 6.0) containing 150 mM NaCl and 2 mM DM was used to collect the 1D ^{1}H spectrum for the receptor. Later, U46619 (100 μM) was added to the sample, which was incubated at 298 K for 20 min, and the 1D and 2D ^{1}H TOCSY spectra were recorded again. All NMR experiments were carried out on a Bruker Avance 800 MHz NMR spectrometer with a 5 mm triple-resonance probe at 298 K. The water peak was suppressed by the excitation sculpting method (*27*). All 1D spectra contain 16K data points. The 2D TOCSY spectra were recorded with an MLEV-17 spin-lock pulse sequence with a total mixing time of 70 ms. Quadrature detection was achieved in F_1 by the States−TPPI method. The NMR data were processed using Felix (Accelrys, San Diego, CA). Shifted sine-bell window functions of 60° for TOCSY were used in both dimensions. Chemical shifts were referenced to the internal standard, DSS (contained in the D_2O), which was set to 0 ppm.

RESULTS

Designing and Subcloning of the Human TP cDNA into the BV Expression Vector. The full size of the human TP cDNA, cloned from human placenta and subcloned into the pcDNA3.1 vector (pcDNA3.1-TP), has been previously reported (*25*). The TP protein, expressed in the COS-7 and HEK293 mammalian cells, using the constructed pcDNA3.1-TP plasmid, demonstrated the production of a full-sized and glycosylated TP, with high activity in ligand binding (*25*) and signaling (*23*). It has been confirmed that the addition of six His residues at the C-terminus of the receptor does not affect the biological activity in the mammalian cell expression system (data not shown). Thus, the cDNA subcloned into the BV vector, pVL1392, from the pcDNA3.1-TP plasmid (Figure 1), should also be able to express an active, full-sized TP protein in Sf-9 cells.

Optimization of the Expression of the TP Receptor in Sf-9 Cells Using the BV System. The optimization of the expression of the TP receptor in Sf-9 cells was achieved by several steps, including the use of high-quality Sf-9 cells, purification of the recombinant BV plaque, screening of the different cultures of the Sf-9 cells infected with the different recombinant BV plaques, and the time course investigation, as described below. Since higher expression levels of the recombinant protein were often observed by the use of cultured Sf-9 cells with no more than 30 passages, this was applied to the TP expression. In the initial expression studies, 10 individual cultures of the high-quality Sf-9 cells were infected with 10 individually purified recombinant BV plaques, which contained the TP cDNA. The different cultures of the Sf-9 cells were able to express the glycosylated TP receptor with different expression yields, as demonstrated by the Western blot (Figure 2A) and corresponding ligand binding assays (Figure 2B). Culture C of the Sf-9 cells exhibited a good expression yield and the highest ligand binding activity (Figure 2); thus, it was selected for further small-scale expression to determine the better harvesting time, where the expressed TP receptor was once again compared by Western blot and ligand binding assays. The studies revealed that the expression yield of the TP receptor reached a maximum at 64 h and had no significant changes up to 72 h (data not shown). The expression yield of the receptor, in Sf-9 cells, at 64 h is approximately 100-fold higher than the expression yield in COS-7 cells using the pcDNA3.1 vector, as evidenced by Western blot analysis (Figure 3A) and the ligand binding activity (Figure 3B). These data strongly suggest that even though a large amount of the recombinant TP protein was produced in the Sf-9 cells, most of the receptor protein has protein folding similar to that of the TP protein expressed in COS-7 cells, where it retains native ligand binding activity.

Purification of the TP Receptor. The TP expressed in the Sf-9 cells was extracted by different detergents, including Triton X-100 (data not shown), Tween 20 (data not shown), OG, and DM. All of the detergents could extract the TP protein from the cell membrane and maintain the receptor−

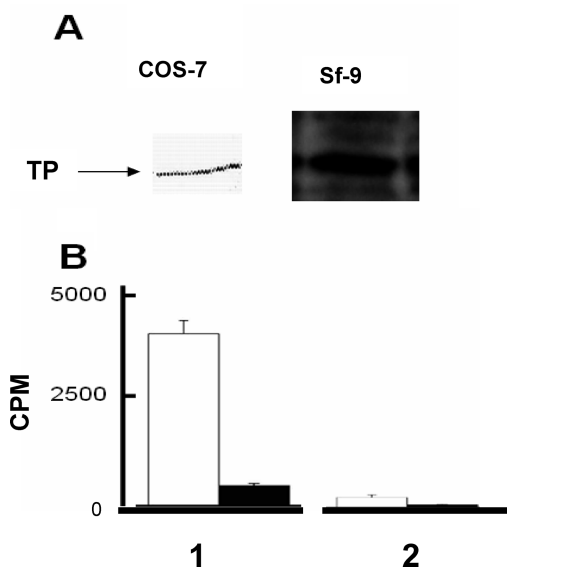

FIGURE 3: Comparison of the expression levels of the human TP in COS-7 and Sf-9 cells. (A) Western blot analysis of the recombinant TP expressed in COS-7 cells and Sf-9 cells. (B). Binding of Sf-9 cells (1) and COS-7 cells (2) expressing TP protein to [^{3}H]SQ29,548 in the absence (white bars) and presence (black bars) of 1.0 μM unlabeled SQ29,548. The experimental conditions are described in the legend of Figure 2.

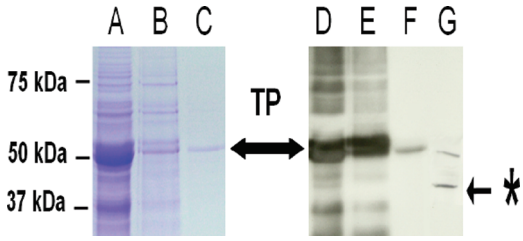

FIGURE 4: Purification of the TP using *n*-octyl β-D-glucopyranoside (OG). The purified TP was subjected to SDS−PAGE on a 10% polyacrylamide gel and then analyzed by protein staining (A−C) and Western blotting (D−G). (A and D) Solubilized TP from the Sf-9 cells using 1% OG. (B and E) FPLC-purified TP. (C and F) Ni column-purified TP. (G) Deglycosylated TP digested with peptide:*N*-glycosidase F (PNGase F). The deglycosylated TP is indicated with an asterisk.

ligand binding activity. The detergent OG, which had successfully been used in the purification of many membrane proteins for structural studies (*28–30*), was selected for the TP purification first. Figure 4 shows the different purity levels of the TP protein purified after OG extraction and removal of the insoluble membrane proteins using ultracentrifugation (Figure 4A,D), further FPLC purification with the Superdex column (Figure 4B,E), and the final Ni column affinity purification (Figure 4C,F). The purified receptor retained high ligand binding activity as confirmed by [^{3}H]SQ29,548 binding assays at each step of the purification. At the final elution from the Ni column, approximately 0.2 mg of the purified TP with a specific ligand binding ratio of 1:0.9 $\pm$ 0.05 ([^{3}H]SQ29,548:TP molecule) was obtained from the original 500 mL of Sf-9 cells with high-yield expression of the TP.

Post-Translational Modification of the Receptor. The overexpressed TP in the transfected mammalian cells, such as HEK293 and COS-7 cells, showing different degrees of glycosylation has been previously reported (*17, 20, 31*). The molecular mass of the TP polypeptide without post-translational modification is expected to be 35−37 kDa (*32*). However, in human platelets, the active TP receptor with full post-translational modification has a molecular mass of approximately 50−51 kDa (*31*). The overexpressed TP receptor in the Sf-9 cells exhibited a single band at approximately 51 kDa (Figures 3 and 4), which strongly suggested that the receptor has a post-translational modification similar to that of the native platelet TP. The single band at the 51 kDa position also indicated that most of the overexpressed receptor molecules are glycosylated. The glycosylation of the expressed TP was further confirmed by a deglycosylation experiment using Western blot analysis (Figure 4G). These results provide evidence that the purified TP, from the Sf-9 cell expression, is suitable for the characterization of the full-sized TP with glycosylation.

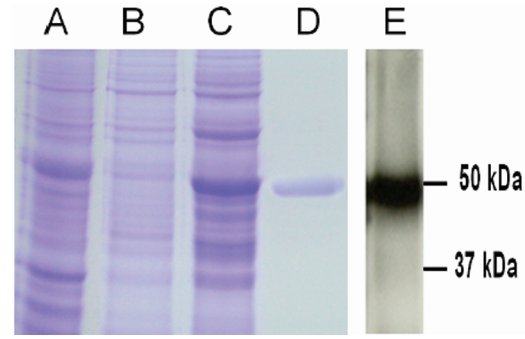

FIGURE 5: Yield comparison of the solubilized receptor from the membrane fraction using OG and DM. The TP that was purified using OG (A and C) and DM (B and D) after ultracentrifugation (A and B) and FPLC purification (C and D) underwent SDS−PAGE analysis, and their protein stainings were compared. The Western blot for the purified TP using DM is shown in lane E. The amount for lanes A and C is approximately 20 μg, and it is approximately 10 μg of TP protein for lanes B, D, and E.

A Simple, Quick, and High-Yield Purification of the TP Receptor. As mentioned above, no solution or crystal structures have been made available for the full-sized prostanoid receptors so far. These studies address the question of how to prepare a sufficient amount of TP protein for structural studies, especially for the solution structure determination using high-resolution NMR spectroscopy. One of the major problems in generating membrane proteins for the NMR studies is the difficulty in obtaining a large amount of active receptor with ^{15}N and ^{13}C labeling because the isotope labeling medium is too expensive to be used for Sf-9 cell expression if the purification yield is not high enough. Thus, increasing the purification yield and decreasing the use of isotope labeling medium could advance the studies. Therefore, we began to focus on the development of a simple, quick, and high-yield purification approach for the TP by optimizing the aforementioned purification conditions. First, the detergent (OG) that was used for the purification was replaced with the DM detergent to more effectively extract the TP protein from the cells. Surprisingly, the purity of the DM-extracted TP reached approximately 20% of the total protein (Figure 5B) following the removal of insoluble membrane proteins by ultracentrifugation, which was significantly higher than that of the OG-extracted TP (Figure 5A). However, the DM-extracted TP protein was contaminated with DNA as evidenced by UV and CD spectroscopic analyses (data not shown). To minimize the DNA contamination, DNase was simply used to break down the DNA into

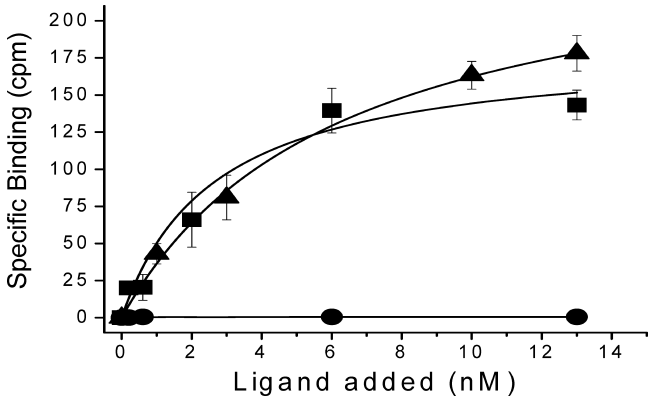

FIGURE 6: Comparison of the specific ligand binding activity between the purified TP from the Sf9 cells and the wild-type TP expressed in COS-7 cells. The purified TP [approximately 20 μg/tube (▲)] derived from Sf9 cells or the original COS-7 cells expressing TP protein [400 μg/tube (■)] in PBS were incubated with increasing amounts of the ligand ([^{3}H]SQ29,548) from 0 to 13 nM. The unlabeled SQ29,548 (1 μM) was added to the COS-7 cells as a negative control (●) before the labeled ligand was added. The results are representative data from three assays ($n = 3$) and are shown as means ± the standard error.

small pieces. We found that the optimized FPLC purification, using the Superdex-75 column with a slow flow rate, was very effective in further separating the DM-solubilized TP protein from other membrane proteins as well as the broken-down DNA fragments. The resultant purified TP protein from these simple and quick procedures exhibited a nearly single major band in SDS−PAGE (Figure 5D) and Western blot analyses (Figure 5E), with a specific ligand binding ratio of 1:0.8 ± 0.05 ([^{3}H]SQ29,548:TP molecule). The final yield of the purified TP protein was approximately 4 mg from the original 500 mL of Sf-9 cells. The purification yield was increased by 20-fold in comparison to that of the affinity purification described in Figure 3 using the Ni column, which suggests that its purity was only slightly decreased (from 1:0.9 ± 0.05 to 1:0.8 ± 0.05) in terms of the molecular ratios of the bound ligand to the purified protein.

Comparison of the Ligand Binding Affinity between Purified TP and the Wild-Type TP Expressed in COS-7 Cells. The TP protein, purified by the high-yield purification method, was further investigated for its binding affinity in comparison to that of the wild-type TP expressed in COS-7 cells using [^{3}H]SQ29,548. Two concentration-dependent curves displaying the results from this comparison are shown in Figure 6. The binding of the purified TP has a K_d value (approximately 6 nM) which is very similar to that of the wild-type TP expressed in COS-7 cells (Figure 6). The results indicated the ligand binding affinity of the TP was not significantly reduced by the nonionic detergent (DM) extraction and the purification processes. Furthermore, the results suggest that the protein folding and conformation of the purified TP are similar to those of the wild-type TP expressed in COS-7 cells.

Feasibility of the Purified Protein for Structural Studies Using NMR Spectroscopy. NMR spectroscopic signals for the purified and active prostanoid receptors have never been attempted because the milligram range of the purified receptors was never available in the past. With 4 mg of the purified TP prepared from 500 mL of the Sf-9 cell culture, we have been able to test whether the proton (^{1}H) signals of

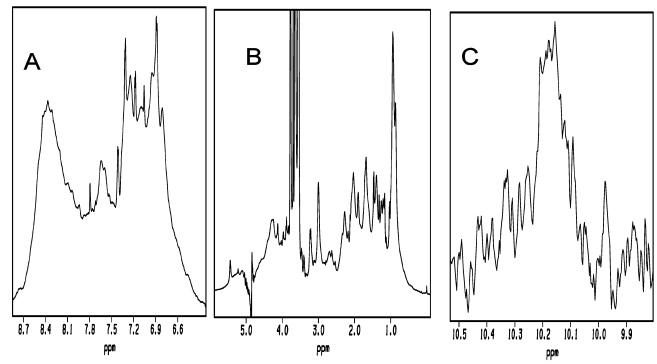

FIGURE 7: Expanded regions for the 1D ^{1}H NMR analysis of the purified TP protein. The 1D spectrum of the purified TP protein (4 mg/0.5 mL) was recorded by the 800 MHz NMR instrument in the presence of U46619 (100 μM) in the buffer (pH 6.0) containing 2 mM DM as described in Experimental Procedures. The expanded 1D NMR spectra for the regions of the amide protons (A), side chain protons (B), and Trp indole ring protons (C) of the U46619-bound and purified TP are displayed.

the receptor protein in DM can be observed. This information can be very useful in determining the next experiment, which consists of the preparation of ^{15}N- and ^{13}C-labeled TP. We have found that the proton signals of the purified TP could be enhanced by the addition of ligand to the protein in the initial 1D test (data not shown). The 1D ^{1}H signals were successfully observed for the purified TP at a concentration of 4 mg/0.5 mL in the presence of 100 μM U46619 using an 800 MHz high-resolution NMR instrument (Figure 7). The main chain amide protons within the 6.5−8.7 ppm range (Figure 7A) and side chain protons within the 0.9−6 ppm range (Figure 7B) have strong signals even though they are overlapped. Surprisingly, even the weak indole ring protons within the 9.8−10.5 ppm range (Figure 7C) were also able to appear in the spectrum. The general spin system distribution of the TP amino acid residues was further observed in the 2D TOCSY spectrum (Figure 8). The proton cross-peaks of the NH/αH region (Figure 8A) and the side chain protons (Figure 8B) were clearly visible. Several residue types of the active TP protein were easily identified in the 2D TOCSY spectrum (Figure 8). For example, as described above, a group of proton peaks in the 1D spectrum within the 9.8−10.5 ppm range, belonging to the nine Trp residues, were observed (Figure 7C). In addition, a group of proton cross-peaks of the NH/βH region of Ser residues were also identified (Figure 8A). Also, it should be pointed out that there were no other significant contaminating signals observed in the 2D spectrum. Further testing was performed on the purified receptor to determine its stability under the 2D NMR experimental conditions. Following 3 days of completed 2D NMR experiments, the ligand binding activity of the TP was compared with that of the freshly prepared TP from day 1. Interestingly, the receptor retained approximately 80% of the original activity observed on day 1. The initial spectrum for the purified TP has provided the following important clues for further solution structural determination of the receptor. (1) The quality (in terms of purity) and the quantity of the TP prepared by the simple, quick, and high-yield approach are suitable for high-resolution NMR studies for determining the receptor solution structure. (2) The unlabeled DM (2 mM) did not cause high background levels for the 2D ^{1}H spectrum, which suggests that it is suitable for membrane protein NMR analysis. (3)

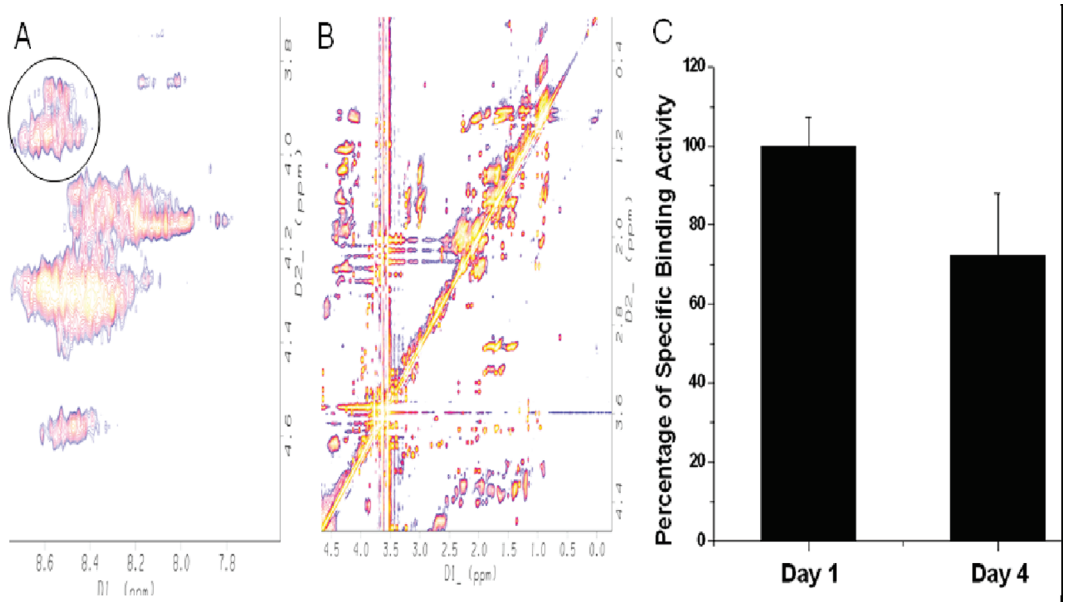

FIGURE 8: Expanded regions of the 2D ^{1}H TOCSY spectrum for the purified TP protein in DM micelles. The spectrum was recorded at 298 K on an 800 MHz NMR spectrometer as described in the legend of Figure 7. The assigned spin systems for the cross-peaks of NH/αH (A) and the side chains (B) of the TP amino acid residues are displayed. The cross-peaks, which were mainly those of the NH/βH region of all the Ser residues in the TP protein, are circled. (C) The purified TP (20 μg/tube) on day 1 (before NMR experiments) and day 4 (after NMR experiments) in PBS was incubated with [^{3}H]SQ29,548 (5 nM). The binding assay was performed as described in the legend of Figure 6. The results are representative data from three assays (n = 3) and are shown as means ± the standard error.

The protein concentration used (4 mg/0.5 mL) is most likely the minimum for obtaining clear proton signals in NMR spectroscopy. (4) The proton NMR experiment suggested that preparation of the isotope-labeled TP protein for solution structural studies can be accomplished with as little as 500 mL of ^{15}N- and ^{13}C-labeled Sf-9 cell culture, which is very practical and feasible.

DISCUSSION

Similar to other GPCR family members, the structural characterization of the full-sized TP has been limited by the lack of high-yield expression and purification systems. In the past, BV expression systems for the TP have been used by different groups (*17, 18, 33*), but the low expression and purification yields were major obstacles keeping the protein from reaching sufficient levels of the pure protein required for structural studies using biophysics approaches. The current studies aim to solve the fundamental problems in the structural studies of the receptor by increasing the expression and purification yields. Our high-yield expression of the full-sized and active TP in Sf-9 cells, using the optimized BV system, can serve as an example of the preparation of large amounts of other full-sized and active prostanoid receptors, such as the DP, IP, and EP receptors. The optimization of the expression of the TP receptor described in the Results, including the purification of the recombinant BV plaques, the use of healthy Sf-9 cells, screening the expression of the different cultures of the Sf-9 cells, and the time course observation are particularly useful for developing a high-yield expression system for the TP. In addition, we have also found that by using these optimized conditions, the high-yield expression system for the TP was highly reproducible. Similar expression yields have been obtained for three trials using the originally selected BV for the Sf-9 cells. The expression levels were also stable at different production scales from 5 to 500 mL of the Sf-9 cell culture.

Here, it was first reported that the full-sized TP receptor, purified via the simple, quick, and high-yield purification, has reached the purity level of nearly a single band in SDS−PAGE and Western blot analysis, with specific ligand binding ratio of 1:0.8 ± 0.05. This high-yield purification was the result of the optimization of the purification conditions via simplification of the purification procedures without significant loss of protein purity. The DM detergent could more effectively extract the TP receptor from the Sf-9 cell membrane and eliminate additional contaminating membrane proteins when compared against other detergents, such as Triton X-100, Tween 20, and OG. The very low critical micelle concentration (CMC, 0.16 mM) and modulate micellar size [approximately 51 kDa (based on 98 aggregation numbers) (*34*)] of DM have made it possible to generate TP protein micelles with significant differences in size and protein concentration. The DM-derived TP protein micelles are suitable for the FPLC purification and NMR spectroscopic analyses in comparison to the TP micelles that were produced by the other commonly used nonionic detergents, which have a high CMC and/or larger micelles. This approach could be suitable for the purification of larger amounts of other human GPCRs for NMR experiments, especially for other prostanoids' receptors, which share significant homologies with the TP receptor. The fact that contaminating DNA, in the initial preparation, could be easily removed by DNase digestion is also an important achievement from this high-yield purification since this could replace other approaches, such as the preparation of the cell membrane, by density gradient centrifugation (to remove the DNA), which could potentially reduce the purification yield.

On the basis of the PAGE and Western blot analysis, the extraction yield of the TP from the Sf-9 cell membrane by

DM is higher than that of OG. Of course, we could not exclude the possibility that the high yield of the DM-solubilized TP may be due to the better stability versus that of OG and less protein degradation versus that of OG. The ligand-to-receptor ratio of 1:0.8 ± 0.05 does not mean that 20% of the protein is inactive. Here are several factors that can be attributed to this ratio. (1) The specific activity of the isotope-labeled ligand is not always 100%. (2) The assay does not always guarantee that 100% binding will be achieved. (3) Handling the purified protein at room temperature and/or the freeze−thaw process may also affect the binding activity. From the original 500 mL of Sf-9 cell infection to the 2D ^{1}H NMR spectrum for the TP described here, this was an initial trial showing the feasibility for the structural determination of the TP receptor using biophysics approaches. It was not an attempt to determine the TP solution structure using the unlabeled TP purified protein because aside from the needs of an advanced NMR instrument and a high-milligram range of purified proteins, NMR structural determination of the membrane proteins also requires ^{15}N and/or ^{13}C labeling of the entire protein as well as a low background from the detergent micelles. The ^{1}H spectra exhibiting peaks around 10 ppm represent the indole side chains of the Trp residues in the TP protein. The signals of the protons in the spectra could be further improved by increasing the protein concentration and ^{15}N or ^{13}C labeling of the Trp residues, which are both priorities on our list of future works that aim to improve our understanding of the solution conformation of the receptor by using the developed purification approach.

Obtaining NMR spectra in the past, with uniform labeling of a glycosylated GPCR, was likely to require large volumes (approximately 3−6 L) of special cell culture medium containing the ^{15}N- and ^{13}C-labeled amino acids or compounds that can be converted into amino acids. This can be unreasonably costly, especially using the Sf-9 cell culture. This study makes a great impact in solving the fundamental issues regarding the structural characterization of the TP. The fact that proton NMR spectra for the TP could be determined, using the purified membrane protein from the Sf-9 cell culture, strongly suggested that the GPCR could be uniformly labeled with the initial use of as little as 500 mL of Sf-9 cell culture, which is 6−12-fold smaller than the volume of the labeling medium that would be required for the Sf-9 cell expression systems of the previously reported methods. Therefore, this has made the labeling option more feasible in the preparation of the membrane protein for the solution structural determination. It shall be indicated that the high-yield expression of the TP, using serum-free Sf-9 cell culture medium containing ^{15}N- and/or ^{13}C-labeled amino acids, was recently initiated and is still being optimized and remains a high-cost experiment at this stage. Thus, the 3D NMR experiment could not be included in this study. However, the milligram range of the purified TP protein that was obtained has established not only an opportunity for the NMR experiments but also the likelihood for an initial attempt at crystallization of the receptor using unlabeled TP protein.

Recently, the NMR spectroscopic technique has been recognized as one of the most powerful tools for characterizing the structural and functional relationship of GPCRs in solution (*35–39*). For example, a detergent-solubilized rhodopsin protein that is suitable for solution NMR spectroscopy has been reported (*38, 39*). The differential dynamics of the rhodopsin protein in solution were revealed by solution NMR (*39*). In addition, solid-state NMR spectroscopy has become another powerful tool for characterizing intact GPCR structures and their interaction with ligands (*40–44*). It has been well documented that a majority of solid-state NMR methods prefer to have receptors at the milligram level. Thus, obtaining a milligram amount of the full-length TP has provided a basis for exploring solid-state NMR spectroscopy for further characterization of the receptor structure and function. For example, the purified TP receptor could be reconstituted into lipid vesicles for the solid-state NMR studies. As a conclusion, this study provides the necessary insight for paving the way toward obtaining structural information about the prostanoid receptors using the NMR spectroscopic technique.

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REFERENCES

1. Majerus, P. W. (1983) Arachdonate metabolism in vascular disorders. *J. Clin. Invest. 72*, 1521–1525.
2. Samuelson, B., Goldyne, M., Granstrom, E., Hamberg, M., Hammarstrom, S., and Malmsten, C. (1978) Prostaglandins and thromboxanes. *Annu. Rev. Biochem. 47*, 994–1030.
3. Francois, H., Athirakul, K., Mao, L., Rockman, H., and Coffman, T. M. (2004) Role for thromboxane receptors in angiotensin-II-induced hypertension. *Hypertension 43*, 364–369.
4. Onguru, O., Casey, M. B., Kajita, S., Nakamura, N., and Lloyd, R. V. (2005) Cyclooxygenase-2 and thromboxane synthase in non-endocrine and endocrine tumors: A review. *Endocr. Pathol. 16*, 253–277.
5. Hirata, M., Hayashi, Y., Ushikubi, F., Yokata, Y., Kageyama, R., Nakanishi, S., and Narumiya, S. (1991) Cloning and expression of cDNA for a human thromboxane A_2 receptor. *Nature 349*, 617–620.
6. Raychowdhury, M. K., Yukawa, M., Collins, L. J., McGrail, S. H., Kent, K. C., and Ware, J. A. (1994) Alternative splicing produces a divergent cytoplasmic tail in the human endothelial thromboxane A2 receptor. *J. Biol. Chem. 269*, 19256–19261.
7. Hirata, T., Ushikubi, F., Kakizuka, A., Okuma, M., and Narumiya, S. (1996) Two thromboxane A2 receptor isoforms in human platelets. Opposite coupling to adenylyl cyclase with different sensitivity to Arg60 to Leu mutation. *J. Clin. Invest. 97*, 949–956.
8. Boie, Y., Sawyer, N., Slipetz, D. M., Metters, K. M., and Abramovitz, M. (1995) Molecular cloning and characterization of the human prostanoid DP receptor. *J. Biol. Chem. 270*, 18910–18916.
9. Funk, C. D., Furci, L., FitzGerald, G. A., Grygorczyk, R., Rochette, C., Bayne, M. A., Abramovitz, M., Adam, M., and Metters, K. M. (1993) Cloning and expression of CDNA for the human prostaglandin E receptor EP1 subtype. *J. Biol. Chem. 268*, 26767–26772.
10. An, S., Yang, J., Xia, M., and Goetzl, E. J. (1993) Cloning and expression of the EP2 subtype of human receptors for prostaglandin E_2. *Biochem. Biophys. Res. Commun. 197*, 263–270.
11. Adam, M., Boie, Y., Rushmore, T. H., Muller, G., Bastien, L., McKee, K. T., Metters, K. M., and Abramovitz, M. (1994) Cloning and expression of three isoforms of the human EP_3 prostanoid receptor. *FEBS Lett. 338*, 170–174.
12. Regan, J. W., Bailey, T. J., Donello, J. E., Pierce, K. L., Pepperi, D. J., Zhang, D., Kedzie, K. M., Fairbaim, C. D., Bogardus, A. M., Woodward, D. F., and Gil, D. W. (1994) Molecular cloning and

expression of human EP_3 receptors: Evidence of three variants with differing carboxyl termini. *Br. J. Pharmacol. 112*, 377–385.
13. Kunapuli, S. P., Mao, G. F., Bastepe, M., Liu-Chen, L. Y., Li, S., Cheung, P. P., DeRiel, J. K., and Ashby, B. (1994) Cloning and expression of a prostaglandin E receptor EP_3 subtype from human erythroleukaemia cells. *Biochem. J. 298*, 263–267.
14. Yang, J., Xia, M., Goetzl, E. J., and An, S. (1994) Cloning and expression of the EP_3-subtype of human receptors for prostaglandin E_2. *Biochem. Biophys. Res. Commun. 198*, 999–1006.
15. Abrarnovitz, M., Boie, Y., Nguyen, T., Rushmore, T. H., Bayne, M. A., Metters, K. M., Slipetz, D. M., and Grygorczyk, R. (1994) Cloning and expression of a cDNA for tile human prostanoid FP receptor. *J. Biol. Chem. 269*, 1356–1360.
16. Katsuyama, M., Sugimoto, Y., Namba, T., Irie, A., Negishi, M., Narumiya, S., and Ichikawa, A. (1994) Cloning and expression of a cDNA for the human prostacyclin receptor. *FEBS Lett. 344*, 74–88.
17. Chiang, N., and Tai, H. H. (1998) The Role of N-Glycosylation of Human Thromboxane A_2 Receptor in Ligand Binding. *Arch. Biochem. Biophys. 352*, 207–213.
18. Chiang, N., Kan, W. M., and Tai, H. H. (1996) Site-directed mutagenesis of cysteinyl and serine residues of human thromboxane A2 receptor in insect cells. *Arch. Biochem. Biophys. 334*, 9–17.
19. Zhou, H., Yan, F., and Tai, H. H. (2001) Phosphorylation and desensitization of the human thromboxane receptor-α by G protein-coupled receptor kinases. *J. Pharmacol. Exp. Ther. 298*, 1243–1251.
20. Pawate, S., Schey, K. L., Meier, G. P., Ullian, M. E., Mais, D. E., and Halushka, P. V. (1998) Expression, characterization, and purification of C-terminally hexahistidine-tagged thromboxane A2 receptors. *J. Biol. Chem. 273*, 22753–22760.
21. Wu, J., Feng, M., and Ruan, K. H. (2007) Assembling NMR structures for the intracellular loops of the human thromboxane A_2 receptor: Implication of the G protein-coupling pocket. *Arch. Biochem. Biophys. 470*, 73–82.
22. Ruan, K. H., Wu, J., So, S. P., Jenkins, L. A., and Ruan, C. H. (2004) NMR structure of the thromboxane A2 receptor ligand recognition pocket. *Eur. J. Biochem. 271*, 3006–3016.
23. Geng, L., Wu, J., So, S. P., Huang, G., and Ruan, K. H. (2004) Structural and functional characterization of the first intracellular loop of human thromboxane A2 receptor. *Arch. Biochem. Biophys. 423*, 253–265.
24. Wu, J., So, S. P., and Ruan, K. H. (2003) Solution structure of the third extracellular loop of human thromboxane A2 receptor. *Arch. Biochem. Biophys. 414*, 287–293.
25. So, S. P., Wu, J., Huang, G., Huang, A., Li, D., and Ruan, K. H. (2003) Identification of residues important for ligand binding of thromboxane A2 receptor in the second extracellular loop using the NMR experiment-guided mutagenesis approach. *J. Biol. Chem. 278*, 10922–10927.
26. Ruan, K. H., So, S. P., Wu, J., Li, D., Huang, A., and Kung, J. (2001) Solution structure of the second extracellular loop of human thromboxane A2 receptor. *Biochemistry 40*, 275–280.
27. Callihan, D., West, J., Kumar, S., Schweitzer, B. I., and Logan, T. M. (1996) Simple, distortion-free homonuclear spectra of peptides and nucleic acids in water using excitation sculpting. *J. Magn. Reson., Ser. B 112*, 82–85.
28. Urbani, A., and Warne, T. (2005) A colorimetric determination for glycosidic and bile salt-based detergents: Applications in membrane protein research. *Anal. Biochem. 336*, 117–124.
29. Ouellet, M., Falgueyret, J. P., Ear, P. H., Pen, A., Mancini, J. A., Riendeau, D., and Percival, M. D. (2002) Purification and characterization of recombinant microsomal prostaglandin E synthase-1. *Protein Expression Purif. 26*, 489–495.
30. Wallecha, A., and Mishra, S. (2003) Purification and characterization of two β-glucosidases from a thermo-tolerant yeast *Pichia etchellsii*. *Biochim. Biophys. Acta 1649*, 74–84.
31. Mais, D. E., True, T. A., and Martinelli, M. J. (1992) Characterization by photoaffinity labelling of the human platelet thromboxane A2/prostaglandin H2 receptor: Evidence for N-linked glycosylation. *Eur. J. Pharmacol. 227*, 267–274.
32. Kelley, L. P., and Kinsella, B. T. (2003) The role of N-linked glycosylation in determining the surface expression, G protein interaction and effector coupling of the α isoform of the human thromboxane A_2 receptor. *Biochim. Biophys. Acta 1621*, 192–203.
33. Zhang, L., DiLizio, C., Kim, D., Smyth, E. M., and Manning, D. R. (2006) The G12 family of G proteins as a reporter of thromboxane A2 receptor activity. *Mol. Pharmacol. 69*, 1433–1440.
34. Kühlbrandt, W. (1988) Three-dimensional crystallization of membrane proteins. *Q. Rev. Biophys. 21*, 429–477.
35. Cohen, L. S., Arshava, B., Estephan, R., Englander, J., Kim, H., Hauser, M., Zerbe, O., Ceruso, M., Becker, J. M., and Naider, F. (2008) Expression and biophysical analysis of two double-transmembrane domain-containing fragments from a yeast G protein-coupled receptor. *Biopolymers 90*, 117–130.
36. Werner, K., Richter, C., Klein-Seetharaman, J., and Schwalbe, H. (2007) Isotope labeling of mammalian GPCRs in HEK293 cells and characterization of the C-terminus of bovine rhodopsin by high resolution liquid NMR spectroscopy. *J. Biomol. NMR 40*, 49–53.
37. Zheng, H., Zhao, J., Sheng, W., and Xie, X. Q. (2006) A transmembrane helix-bundle from G-protein coupled receptor CB2: Biosynthesis, purification, and NMR characterization. *Biopolymers 83*, 46–61.
38. Loewen, M. C., Klein-Seetharaman, J., Getmanova, E. V., Reeves, P. J., Schwalbe, H., and Khorana, H. G. (2001) Solution ^{19}F nuclear Overhauser effects in structural studies of the cytoplasmic domain of mammalian rhodopsin. *Proc. Natl. Acad. Sci. U.S.A. 98*, 4888–4892.
39. Klein-Seetharaman, J., Yanamala, N. V., Javeed, F., Reeves, P. J., Getmanova, E. V., Loewen, M. C., Schwalbe, H., and Khorana, H. G. (2004) Differential dynamics in the G protein-coupled receptor rhodopsin revealed by solution NMR. *Proc. Natl. Acad. Sci. U.S.A. 101*, 3409–3413.
40. Ratnala, V. R., Kiihne, S. R., Buda, F., Leurs, R., de Groot, H. J., and DeGrip, W. J. (2007) Solid-state NMR evidence for a protonation switch in the binding pocket of the H1 receptor upon binding of the agonist histamine. *J. Am. Chem. Soc. 129*, 867–872.
41. Ratnala, V. R. (2006) New tools for G-protein coupled receptor (GPCR) drug discovery: Combination of baculoviral expression system and solid state NMR. *Biotechnol. Lett. 28*, 767–778.
42. Park, S. H., Prytulla, S., De Angelis, A. A., Brown, J. M., Kiefer, H., and Opella, S. J. (2006) High-resolution NMR spectroscopy of a GPCR in aligned bicelles. *J. Am. Chem. Soc. 128*, 7402–7403.
43. Kiihne, S. R., Creemers, A. F., de Grip, W. J., Bovee-Geurts, P. H., Lugtenburg, J., and de Groot, H. J. (2005) Selective interface detection: Mapping binding site contacts in membrane proteins by NMR spectroscopy. *J. Am. Chem. Soc. 127*, 5734–5735.
44. Luca, S., White, J. F., Sohal, A. K., Filippov, D. V., van Boom, J. H., Grisshammer, R., and Baldus, M. (2003) The conformation of neurotensin bound to its G protein-coupled receptor. *Proc. Natl. Acad. Sci. U.S.A. 100*, 10706–10711.

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