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Characterization of the Cholesterol Recognition Amino Acid Consensus Sequence of the Peripheral-Type Benzodiazepine Receptor

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We previously defined a cholesterol recognition/interaction amino acid consensus sequence [CRAC: L/V–X (1–5)–Y–X (1–5)-R/K] in the carboxyl terminus of the peripheral-type benzodiazepine receptor (PBR), a high-affinity drug and cholesterol-binding protein present in the outer mitochondrial membrane protein. This protein is involved in the regulation of cholesterol transport into the mitochondria, the rate-determining step in steroid biosynthesis. Reconstituted wild-type recombinant PBR into proteoliposomes demonstrated high-affinity 2-chlorophenyl)-*N*-methyl-*N*-(1-methyl-propyl)-3-isoquinolinecarboxamide and cholesterol binding. In the present work, we functionally and structurally characterized this CRAC motif using reconstituted recombinant PBR and nuclear magnetic resonance. Deletion of the C-terminal domain of PBR and mutation of the highly conserved among all PBR amino acid sequences Y152 of the CRAC domain resulted in loss of the ability of mutant recPBR to bind cholesterol. Nuclear magnetic resonance analysis of a PBR C-terminal peptide (144–169) containing the CRAC domain indicated a helical conformation for the L144–S159 fragment. As a result of the side-chain distribution, a groove that could fit a cholesterol molecule is delineated, on one hand, by Y152, T148, and L144, and, on the other hand, by Y153, M149, and A145. The aromatic rings of Y152 and Y153 assigned as essential residues for cholesterol binding constitute the gate of the groove. Furthermore, the side chain of R156 may cap the groove by interacting with the sterol hydroxyl group. These results provide structural and functional evidence supporting the finding that the CRAC domain in the cytosolic carboxyl-terminal domain of PBR might be responsible for the uptake and translocation of cholesterol into the mitochondria. (*Molecular Endocrinology* 19: 588–594, 2005)

THE PERIPHERAL-TYPE BENZODIAZEPINE receptor (PBR) was originally discovered because it binds the benzodiazepine diazepam with relatively high affinity (1–3). Subsequent studies demonstrated that PBR binds a variety of distinct chemical entities, including isoquinoline carboxamides, imidazopyridines, indole derivatives, pyrrolobenzoxazepines, phenoxyphenyl acetamide derivatives, and others (4). Among these compounds, the isoquinoline carboxamide, 1-(2-chlorophenyl)-*N*-methyl-*N*-(1-methyl-propyl)-3-isoquinoline carboxamide (PK 11195), has been the most widely used diagnostic of PBR presence and function (2, 5, 6). PBR, although present in all tissues examined, was found to be particularly high in steroid- and bile acid-producing tissues, where it was localized primarily in the outer mitochondrial membrane (2, 7). Since its discovery, this 18-kDa hydrophobic protein has been involved in various cell functions, including steroidogenesis, cell proliferation, mitochondrial respiration, and apoptosis (4–6). From these studies, its role in steroid biosynthesis provided the first clue to its putative function (2, 7).

In various cell systems and isolated mitochondria it was observed that PBR drug ligands stimulate the formation of steroids (2, 7, 8) or bile acids (7, 9) and, more precisely, the transfer of cholesterol from the outer to the inner mitochondrial membrane (10). Targeted disruption of the PBR gene in steroid-synthesizing Leydig cells resulted in the arrest of cholesterol transport into mitochondria and steroid formation;

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Abbreviations: CRAC, Cholesterol recognition amino acid consensus sequence; DPC, dodecyl phosphatidyl choline; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; PBR, peripheral-type benzodiazepine receptor; PK 11195, 1-(2-chlorophenyl)-*N*-methyl-*N*-(1-methyl-propyl)-3-isoquinoline carboxamide; recPBR, recombinant PBR; SDS, sodium dodecyl sulfate; TFE, trifluoroethanol.

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transfection of the PBR-disrupted cells with a PBR cDNA rescued steroidogenesis (11).

The role of PBR in cholesterol transport was further clarified by site-directed mutagenesis and *in vitro* expression studies showing that a region of the cytosolic carboxyl terminus of the receptor contains a cholesterol recognition amino acid consensus sequence [CRAC: L/V–X (1–5)–Y–X (1–5)–R/K] (12, 13). This motif has been found in other transmembrane proteins that interact with cholesteryl groups (12, 14).

The first direct demonstration that PBR is a high-affinity cholesterol-binding protein came from *in vitro* reconstitution experiments of the isolated recombinant protein in proteoliposomes unveiling that PBR binds drug ligands and cholesterol with low nanomolar affinity (15).

Analysis of the cDNA sequences from different mammals indicated that the 18-kDa PBR protein contained 169 amino acids with a high degree of identity and homology among species (2, 5, 6). Based on hydropathy profile analysis of the amino acid sequence, a putative five-transmembrane structure has been proposed (16–18) with the carboxyl terminus exposed to the cytoplasm as established by topological analysis (16). Therefore, the cholesterol recognition amino acid sequence is located at the C terminus of the last transmembrane domain.

The aim of the present work was to further characterize, from both a functional and structural point of view, the recognition of cholesterol by the PBR, the first event in the transport. We report herein site mutagenesis experiments performed by a complete analysis of the ligand-binding properties of deleted C terminus and Y152 mutant reconstituted recombinant PBR (recPBR) into proteoliposomes. These results combined with nuclear magnetic resonance (NMR) structural data and molecular modeling of cholesterol binding to the carboxyl-terminal domain of PBR demonstrate that the carboxyl-terminal segment of PBR constitutes an essential part of the cholesterol-binding domain.

RESULTS

Reconstitution of recombinant mouse PBR into proteoliposomes results in a functional receptor protein maintaining its ability to bind both the PBR drug ligand, PK 11195, and cholesterol with nanomolar affinities (15, 22). The pharmacological characteristics of the reconstituted recPBR were similar to those reported for the native mouse protein (8). Site-directed mutagenesis of the C terminus of PBR was shown previously to dramatically reduce the cholesterol uptake function of the receptor expressed in *Escherichia coli* proteoplasm, whereas it retained its capacity to bind PK 11195 (12). It was also shown that Y153 and R156 were involved in the interaction of PBR with cholesterol. Considering these results and our progress in reconstituting a functional wild-type recPBR (15), we examined the function of recPBR protein in which the C terminus was deleted. Figure 1 shows that the C terminus-deleted protein reconstituted in liposomes loses almost all of its ability to bind both drug ligand PK 11195 (*left*) and cholesterol (*right*) compared with the wild-type PBR. To further characterize the cholesterol-binding site, we performed further site-directed mutagenesis. For this study, we chose to mutate Y152, which is the most highly conserved among all mammalian PBR sequences tyrosine residues. Figure 1 shows that the reconstituted mutant

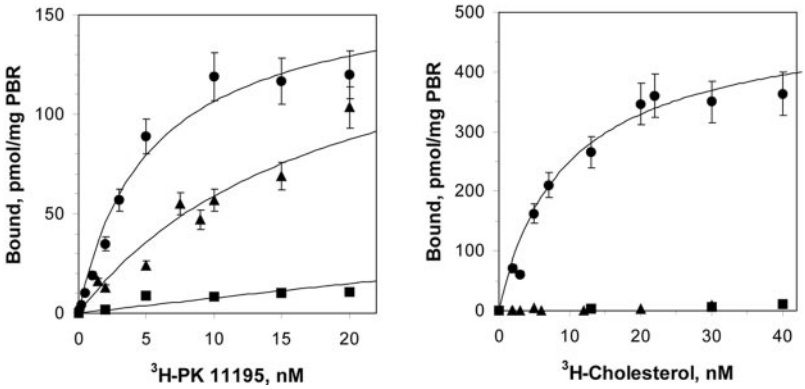

Fig. 1. Saturation Isotherms of [^{3}H]PK 11195 and [^{3}H]Cholesterol Binding to Reconstituted recPBR

Reconstitution of mouse PBR protein at a 5:1 (wt/wt) lipid to protein ratio was performed as described in *Materials and Methods* using a mixture of dimyristoyl phosphatidyl choline/dimyristoyl phosphatidyl ethanolamine (9:1). Binding experiments using [^{3}H]PK 11195 (*left*) and [^{3}H]cholesterol (*right*) were performed with wild-type (*circles*), Y152S mutant (*triangles*), and C terminus-deleted PBR (*squares*) as described in *Materials and Methods*. Saturation curves are shown, and the results of the subsequent Scatchard analyses for PK 11195 binding were: $K_d = 4.5 \pm 0.5$ and 17 ± 4 nM; $B_{max} = 135 \pm 5$ and 135 ± 5 pmol/mg; and nH = 0.95 ± 0.025 and 0.95 ± 0.02 for wild-type and Y152S mutant PBRs, respectively. Scatchard analysis for cholesterol binding resulted in $K_d = 10 \pm 0.5$ nM; $B_{max} = 450 \pm 20$ pmol/mg; and nH = 0.95 ± 0.01. Results are representative of three independent experiments (reconstitutions) performed in triplicate.

(Y152S) conserved its binding properties for the drug ligand PK 11195 (*left*), whereas it lost completely its ability to bind cholesterol (*right*). Interestingly, mutation of Y152 into S slightly reduced the affinity of the protein for PK 11195 [dissociation constant (K_d) was increased from 4.5 to 17 nM] without changing the maximum number of binding sites (B_{max}), suggesting that this residue might not be involved in the binding of the PBR drug ligand. Conversely, the loss in cholesterol binding suggests that this residue is involved in the interaction of the receptor with cholesterol. Considering the previous data obtained with other mutants (Y153S and R156L), which also showed similar results, we decided to perform NMR structural analysis of the peptide spanning the C-terminal region of the rat PBR sequence. Residues predicted to form three helix turns of the putative fifth transmembrane helix were included in the peptide sequence Ac–L–A_{145}–F–A–T–M–L_{150}–N–Y–Y–V–W_{155}–R–D–N–S–G_{160}–R–R–G–G–S_{165}–R–L–T–E.

In a first set of experiments, the PBR peptide was tentatively solubilized in the presence of either perdeuterated dodecyl phosphatidyl choline (DPC) or sodium dodecyl sulfate (SDS) micelles, currently used as membrane-like environments for NMR studies of membrane peptides. As judged by the signal to noise ratio, addition of DPC micelles only partially solubilized the peptide (Fig. 2A). For unclear reasons and regardless of the DPC concentration, the resolved resonances corresponded to only 10% of the expected proton signals. In the presence of SDS instead of DPC, the PBR peptide was fully solubilized but, as shown in Fig. 2B, it resulted in a narrow spectral dispersion of the amide proton signals, indicating a poorly structured conformation. Fortunately, using a trifluoroethanol (TFE) solution we obtained both a full solubilization and spectral dispersion characteristic of highly structured peptides (Fig. 2C). Figure 2 also shows that addition of TFE induces a spectacular high-field shift of the W155 indole NH resonance up to overlap an amide proton signal.

Proton assignment of the C-terminal PBR fragment solubilized in deuterated TFE was achieved using standard total correlated spectroscopy and nuclear Overhauser effect (NOE) spectroscopy experiments (20). Figure 3A summarizes the sequential and medium range NOEs involving the backbone protons. A set of intense NH–NH (i,i+1) NOEs associated with a continuous network of CαH–NH (i,i+3) and (i,i+4) contacts indicates the formation of a unique helix from the N terminus of the peptide up to N158. In the remaining part of the peptide backbone, no medium range NOEs network was detected, indicating that this region does not adopt any predominant conformation. Examination of the Hα chemical shift indexes ($\Delta\delta$Hα) of the PBR peptide (Fig. 3B**)** confirmed the NOE analysis. These results suggest a propensity to form an helical conformation in the C-terminal region from S159. The presence of a central patch of aromatic residues (Y152–Y153–V–W155) explains the rather erratic $\Delta\delta$Hα profile obtained for the PBR peptide.

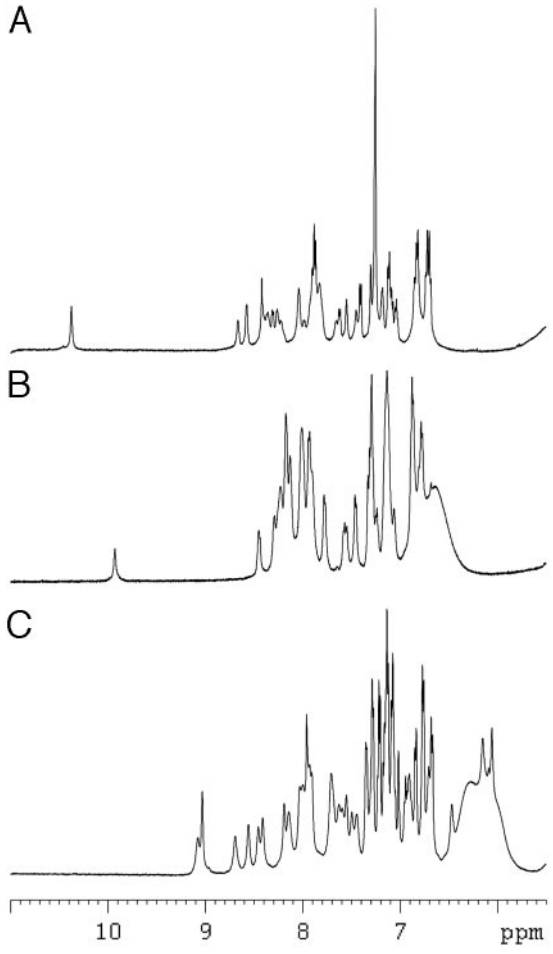

Fig. 2. Amide and Aromatic Proton Signals from 500 MHz [^{1}H]NMR Spectra of the PBR Peptide Containing the CRAC Domain Solubilized in Different Membrane-Like Environments

A, DPC-d38 micelles; B, SDS-d25 micelles; and C, a pure TFE-d2 solution. The peptide concentration was 2 mM and the temperature 303 K.

In a second step, molecular modeling of the PBR sequence L144–G164 was performed using NOEs as distance constraints. As expected from the characteristic NOE network, the structures obtained from simulated annealing and minimization cycles exhibited a helical conformation in the N-terminal part of the peptide. The interesting information provided by the peptide modeling mainly concerns the C-terminal capping interactions, on the one hand, and the average positioning of specific side chains on the other hand. The refined description of the helical structure obtained from the 10 molecules selected for their lowest energy highlights the following features: 1) the N-terminal segment of the PBR peptide forms three α-helix turns up to W155; 2) the following four polar residues (R–D–N–S) constitute a last helix turn characterized by a C-capping i,i+3 hydrogen bond between NH(S159) and CO(R156); 3) for half of the lowest energy struc-

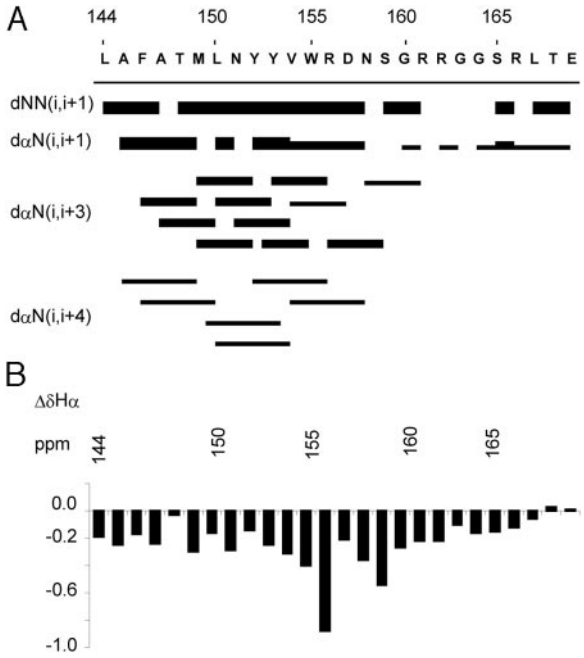

Fig. 3. Summary of the Proton NMR Data for the PBR Peptide Containing the CRAC Domain (2 mM in TFE-d2, 303 K).

A, Sequential and medium range NOEs involving the backbone protons of the PBR peptide. The *bar thickness* refers to the NOE intensity (strong, medium, weak). B, Chemical shift indexes ($\Delta\delta H\alpha$).

tures, this classical 3_{10} helix termination is strengthened by a OH(S159)–CO(W155) side chain to backbone H-bond and an additional i,i+3 NH(G160)–CO(D157) backbone to backbone H-bond; and 4) for all the structures, a R161–D157 salt bridge stabilizes the capping motif, which can be supplemented by a R162–N158 interaction.

In summary, using the standard nomenclature, the N-terminal part of the PBR C-terminal peptide containing the CRAC domain adopts a well-defined helical structure in which S159 and G160 constitute the C-cap and C′ residues, respectively (21). The C″ and C‴ residues, R161 and R162, are also involved in stabilizing the capping structure.

Another set of interesting structural features concerns the average conformation of several side chains. First, the indole group of W155 is found preferentially interacting with the amide group of the N151 side chain. This H bond was expected from NOE data (data not shown) and explains the spectacular high-field shift observed for the W155 indole NH signal, $\delta = 9.01$ ppm, which corresponds to a $\Delta\delta$ greater than -1 ppm with respect to a W residue in a coil conformation (22). As a result, the W aromatic ring is rather parallel to the helix axis with its NH group pointing toward the N terminus. In contrast, the hydroxyl groups of both Y152 and Y153 rings are found pointing toward the helix C terminus to satisfy electrostatic interactions with the R156 and D157 side chains. Therefore, the average orientation of the three aromatic side chains, Y152, Y153, and W155, delineate half a girdle around the helix axis as depicted in Fig. 4. The relative orientation of the W155 and Y152 side chains can be related to the unusual high-field shifts observed for their respective aromatic CH protons (average $\Delta\delta \approx -0.25$ ppm) due to a reciprocal ring current influence. Last, for half of the lowest energy structures, the hydroxyl group of T148 forms an H bond with the carbonyl group of L144. Such an interaction is frequently observed for helices in hydrophobic environments (24).

DISCUSSION

Although the role of PBR in cholesterol transport across the outer mitochondrial membrane has been shown in various steroid- and bile acid-producing tissues, such as gonads, adrenal, brain, placenta, and liver (1–7), and its role in cholesterol compartmentalization and membrane biogenesis in non-steroid-synthesizing tissues has been suggested (2, 7), the exact mechanism by which this protein binds and translocates cholesterol remains poorly understood. In previous studies, we demonstrated that 1) the C-terminal, cytosolic, region of this mitochondrial protein contains a CRAC (12, 13); 2) Y153 and R156 were critical for cholesterol uptake by cells (12, 13); and 3) wild-type recPBR reconstituted into proteoliposomes binds drug ligands and cholesterol with nanomolar affinity (15, 19). To further characterize the interaction of cholesterol with PBR we demonstrated that indeed deletion of the C-terminal domain of the receptor resulted in the complete loss of the ability of recPBR reconstituted in proteoliposomes to bind cholesterol. Based on the conserved amino acid sequence homology across species, we subsequently demonstrated the importance of Y152 in conferring the ability of the

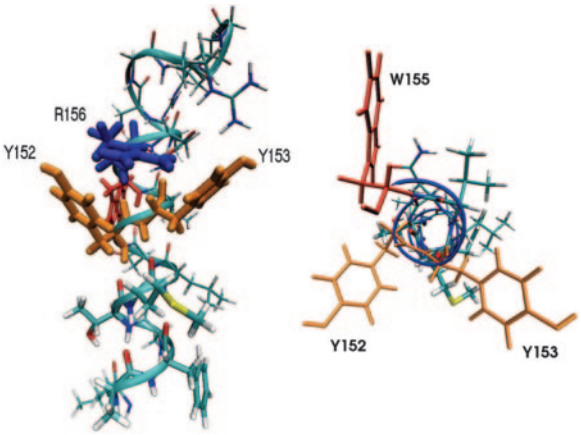

Fig. 4. NMR Model of the PBR Peptide (L144–G164)

Side (*left*) and top (*right*) views. The Y152 and Y153 side chain atoms are colored in *orange*, R156 side-chain atoms are *blue*, and W155 side chain atoms are *red*. The figures were prepared using visual molecular dynamics (23).

receptor to bind cholesterol. These studies suggested that the C-terminal peptide 144–169, facing the cytosol (16), contains most of the critical elements mediating the function of PBR in cholesterol binding and transport.

The C-terminal PBR peptide conformation was analyzed in light of the structure prediction performed on the PBR sequences and, more generally, of our present knowledge of transmembrane helices. According to previously published work (16–18), the last and fifth transmembrane domain of PBR sequences is predicted to form an helix up to W155. It is now well established that W and Y residues are frequently found at the ends of the hydrophobic regions of transmembrane helices and act as interfacial anchors (24, 25). These aromatic residues are preferentially located close to the boundary region separating the hydrophobic core and the membrane interface. Recent emerging pictures of membrane protein structures also point out that transmembrane helices may frequently extend into the lipid head-group region by three or four polar residues involved in capping interactions (24).

Remarkably, our data are fully consistent with the structural features described above. The PBR peptide adopts a unique helix including the predicted last transmembrane residues up to W155, extended by the R156–G160 segment, providing C-capping interactions. Furthermore, a complete interaction network between appropriate side chains gives rise to a girdle of three aromatic residues, Y152, Y153, and W155, which constitutes a strong interfacial anchoring motif.

These conformational properties coupled to the mutagenesis data described above highlight several structural aspects of the cholesterol-binding process. To better appreciate the cholesterol-PBR C-terminal domain interaction, the following aspects must be considered 1) cholesterol is a rather planar molecule, at least as far as the adjacent rings are concerned, thus offering a limited number of conformations; 2) its average length corresponds to about three helix turns and therefore fits the PBR L144–W155 transmembrane domain; and 3) its small polar head, a simple hydroxyl group, should be located at the boundary region of the membrane interface, *i.e*. close to R156. All these features singularly simplify the search of potential docking sites for cholesterol, which, in a first step, can be restricted to examine the occurrence of an appropriate incipient cleft at the L144–W155 helix surface. As a result of the side chain distribution, the hydrophobic surface area around the L144–W155 helix axis is markedly inhomogeneous. Almost all the bulky side chains (F146, L150, V154) are gathered on one helix side, which can thus be ruled out for steric hindrance reasons as a part of a cholesterol binding site. In contrast, the opposite side exhibits a favorable incipient cavity in agreement with docking molecular dynamics (as shown in Fig. 5) and mutagenesis analysis. The corresponding groove is delineated, on one hand, by Y152, T148, and L144, and on the other hand by Y153, M149, and A145. The aromatic rings of Y152 and Y153 assigned as essential residues for cholesterol binding constitute the gate of the groove. Furthermore, the side chain of R156, a residue also found to be critical for cholesterol binding from previous mutagenesis studies (12) and able to form an H bond with Y152 as shown from NMR data, may cap the groove by interacting with the sterol hydroxyl group. A similar fine-tuned electrostatic and hydrophobic interaction network between phosphatidylserine and a single-spanning membrane protein has been recently reported (26).

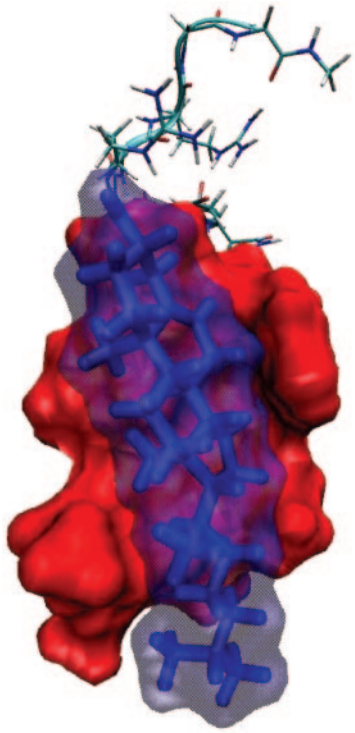

Fig. 5. Docking Model of Cholesterol to PBR Peptide Including the CRAC Domain

The accessible surface of the peptide and cholesterol molecules are represented in *red* and *blue*, respectively. The figure was prepared using visual molecular dynamics (23).

In summary, the combination of functional data obtained on PBR and structural data obtained on the C-terminal domain of this receptor reveals a cholesterol binding concavity and indicates essential residues involved in cholesterol binding. This concavity could define the entrance gate for cholesterol translocation. Comparison of our experimental data with the previously proposed models (17, 18) suggests the involvement of other transmembrane helices to the entrance gate. In particular, facing the CRAC, transmembrane domain 1 could participate in the cholesterol binding site. Therefore, challenging NMR experiments involving the entire receptor and cholesterol-docking simulations in membrane mimetic environments are currently under way to further characterize the cholesterol binding to the receptor.

The presence of a high-affinity binding site for cholesterol suggested that PBR acts as a cholesterol transporter/exchanger rather than a cholesterol channel (7). Thus,

biochemical experiments combined to structural data should contribute to the understanding of the role of PBR in intracellular cholesterol transport at a molecular level.

MATERIALS AND METHODS

Expression and Purification of recPBR

pET15PBR vector was used to transform the BL21(DE3) *E. coli* strain (Novagen, Madison, WI) in which the expression of recombinant mouse PBR protein was induced by 1 mM isopropyl-1-thiol-β-D-galactopyranoside as previously described (12–13, 15). Briefly, two precultures were performed before overexpression was induced by adding 1 mM isopropyl-β-D-thiogalactopyranoside within the exponential growth phase. Cells were harvested, washed in 300 mM NaCl, 50 mM HEPES-NaOH (pH 7.8) (buffer A) and sonicated thoroughly. The pellet was collected at 4000 × *g* centrifugation and dissolved in buffer A containing 0.5% SDS. The solution was centrifuged at 34,000 × *g* to remove unsolubilized material.

recPBR was purified by the His·Bind metal chelation resin (Novagen). Briefly, the supernatant of solubilized inclusion bodies was loaded on Ni-NTA superflow column, which was further washed twice with solution containing detergent and 10 mM imidazole. recPBR elution was obtained by increasing imidazole concentration up to 250 mM. Protein was stored in detergent-containing buffer as previously described (15). This protocol was also used to generate the recombinant mutant proteins described below, with the following modification: a multistep elution process with 10, 20, 30, 40, and 50 mM imidazole was used to eliminate contaminating proteins present in higher levels because of the lower isopropyl-β-D-thiogalactopyranoside-induced expression level of the mutant proteins.

Site-Directed Mutagenesis

Mutations were performed using the QuikChange Site-Directed Mutagenesis kit from Stratagene (La Jolla, CA) as previously described (12, 13). In brief, miniprep pET-PBR plasmid double-strand DNA was used as template. Synthetic oligonucleotide primer pairs containing Y152S point mutation and PBR deletion Δ153–169, each complementary to the opposing strand of the vector, were extended during temperature cycling by *pfu* DNA polymerase. Upon incorporation of the oligonucleotide primers, mutated plasmids containing staggered nicks were generated. After temperature cycling, the products were treated with *Dpn*I to digest the parental DNA template and select for the generated mutation. The nicked vector DNAs containing the desired mutations were then transformed into *E. coli*. The mutated plasmids were prepared by ABI Prism Miniprep Kit. The generated mutations and deletions were confirmed by sequencing using the ABI Prism Dye Terminator Cycle Sequencing ready reaction kit (PerkinElmer Applied Biosystems, Foster City, CA). DNA sequencing was performed at the Lombardi Comprehensive Cancer Center Sequencing Core Facility (Georgetown University).

Reconstitution of recPBR in Liposomes

A stock solution of lipids (dimyristoyl phosphatidyl choline/dimyristoyl phosphatidyl ethanolamine, 9:1) from Avanti Polar Lipids (Alabaster, AL) was mixed with the SDS solubilized isolated recPBR protein at a concentration corresponding to the chosen lipid-to-protein ratio 5:1 (wt:wt). The resulting micellar protein-lipid-detergent mixture was stirred for 15 min at room temperature before addition of Bio-Beads SM2, which removes SDS and induces vesicle formation (15). Bio-Beads were added in a multistep process; first, 1 g Bio-Beads was added per 30 mg SDS and mixed for 60 min. Second and third, the same Bio-Beads amount was added and allowed to stand for another 60 min. Vesicle formation was followed by absorbance at 550 nm. Bio-Beads were subsequently removed and the solution was kept in the cold at 4 C.

Radioligand-Binding Assays

Reconstituted recPBR protein (0.5–2.0 μg/ml) in a 5:1 (wt/wt) lipid to protein ratio was used for PK 11195 and cholesterol ligand-binding studies. [^{3}H]PK 11195 (SA, 83.5 Ci/mmol; NEN Life Science Products, Boston, MA) and [1,2-^{3}H]cholesterol (SA, 43.8 Ci/mmol; NEN Life Science Products) binding studies were performed as we described previously (4, 12). Bound [^{3}H]PK 11195 and [^{3}H]cholesterol were quantified by liquid scintillation spectrometry. K_d values, B_{max} values, and Hill coefficients (nH) for PK 11195 and cholesterol were determined by Curve-Fit (Prism version 3.0, GraphPad Software, Inc., San Diego, CA).

NMR Experiments

The 26-mer peptide (Ac-L–A_{145}–F–A–T–M–L_{150}–N–Y–Y–V–W_{155}–R–D–N–S–G_{160}–R–R–G–G–S_{165}–R–L–T–E) used was chemically synthesized by Biosynthesis Laboratories (Lewisville, TX). Samples were prepared using 2 mM of the PBR peptide solubilized in 90:10 H_2O-D_2O solutions containing 200 mM of either DPC-d_{38} (Cambridge Isotope Laboratories, Cambridge, MA) or SDS-d_{25} (Euriso-Top, Saclay, France) micelles. The pH value was adjusted to 5. In a third set of experiments, the peptide was solubilized in TFE-d_2 (Euriso-Top, Gif sur Yvette, France). [^{1}H]NMR experiments were carried out on DRX 500 and 600 Bruker (Karlsruhe, Germany) spectrometers at various temperatures from 20 to 35 C. Total correlated spectroscopy and nuclear Overhauser effect spectroscopy spectra were recorded with mixing times of 80 msec and 150 msec, respectively. The last release, 6.8, of Sybyl (Tripos, Inc., St. Louis, MO) was used for modeling the PBR fragment. Tripos force field with electrostatics was used for minimization and dynamics. Interresidue distance ranges derived from NOE data were entered as constraints. According to the NOE intensities, the upper limit of proton-proton distance ranges was set to 3, 4, or 5 Å and the lower limit to 1.8 Å. The L144-N158 segment of the starting model was set in a helix conformation and the remaining part was set in a random conformation. The molecule was then subjected to simulated annealing from 800 K to 0 K for 5 psec. One hundred cycles were run. Ten structures were selected from their significantly lower energy. They were then minimized by alternating short low-temperature dynamics (2 psec, 20 K) and minimization runs until the potential energy remained stable.

Cholesterol Docking

The docked location of the cholesterol molecule to the peptide fragment was evaluated by molecular dynamics simulations. The cholesterol molecule was initially positioned 3 Å away from the concavity defined by Y152 and Y153 with its hydrophilic part toward the C terminus. Five molecular dynamics simulations of 4 nsec duration were then carried out for this system without any force constraint applied to the cholesterol molecule. For three of the molecular dynamics simulations, the cholesterol docked inside the concavity previously defined and remained kinetically stable within the concavity during the whole duration of the simulations. This kinetically stable position was further considered as the docking position. All calculations were performed at 300 K using the CHARMM package (27) with CHARMM-22 force-field parameters (28) and cholesterol parameters (29).

Acknowledgments

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