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Site-Directed Mutagenesis of the Peripheral Benzodiazepine Receptor: Identification of Amino Acids Implicated in the Binding Site of Ro5-4864

ROSELI FARGES, EVELYNE JOSEPH-LIAUZUN, DAVID SHIRE, DANIEL CAPUT, GÉRARD LE FUR, and PASCUAL FERRARA
Sanofi Recherche, 31676 Labège Cedex, France
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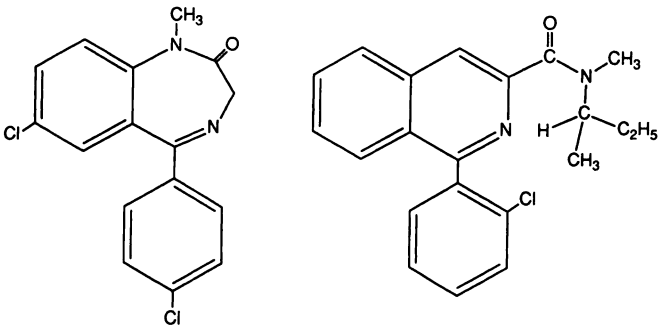
SUMMARY

The peripheral benzodiazepine receptor (PBR) is an 18-kDa protein present in the outer mitochondrial membrane. The human PBR can be labeled with the benzodiazepine Ro5-4864 and with the isoquinoline carboxamide PK11195. The two ligands compete with each other in binding experiments, with previous results suggesting overlapping but not identical binding sites. To define the regions of the receptor interacting with PK11195 and Ro5-4864 and to address the question of the topology of the molecule in the membrane, we generated mutant human PBRs with amino- and carboxyl-terminal deletions and with point mutations in potentially accessible cytoplasmic regions. The mutant genes were expressed in yeast and analyzed in binding experiments using radiolabeled PK11195 and Ro5-4864. The results showed that, whereas deletions in the amino-terminal sequence had marked consequences for the binding affinity of both ligands, the final 13 amino acids at the carboxyl terminus could be deleted with no effect on the binding of either Ro5-4864 or PK11195. The site-directed mutagenesis experiments pinpointed four amino acids as participating in the binding site of Ro5-4864. Three of these, Glu-29, Arg-32, and Lys-39, which are located in the first putative cytoplasmic loop, are conserved in human, bovine, rat, and mouse PBRs. The remaining residue, Val-154, which is found at the interface between the putative fifth transmembrane region and the cytoplasm, is present in the human, rat, and mouse sequences but is replaced by methionine in the bovine sequence. The exchange of Met-154 for valine in the bovine PBR introduced a binding site for Ro5-4864, which is absent in the native PBR. These four amino acids played a minor role, if any, in the binding site of PK11195. We also showed that the histidines previously suggested to be part of the binding site of PK11195 are not directly involved in the interaction of the human receptor with either PK11195 or Ro5-4864.

Benzodiazepines interact with two receptor types, i.e., the central benzodiazepine receptor, associated with the γ-aminobutyric acid-regulated chloride channels located in the central nervous system, where they exert sedative, anxiolytic, and anticonvulsant effects (1), and the PBR, originally described in rat peripheral tissues (2). The PBR is expressed in almost all tissues and is abundantly present in the outer mitochondrial membrane of steroid-producing cells (3–5). In adrenocortical cells the mitochondrial PBR plays a role in the translocation of cholesterol from outer to inner mitochondrial membranes, the rate-limiting step in steroidogenesis (6, 7). A more general regulatory role has been suggested for the PBR present in the glial cells in the brain (8), but no clear function has been ascribed either to the mitochondrial receptor present in non-steroid-producing cells or to the PBR present in the cell membrane (9, 10). The PBR can be labeled with benzodiazepines such as Ro5-4864 and with isoquinoline carboxamides such as PK11195, which are chemically unrelated to benzodiazepines (Fig. 1). Based on thermodynamic studies of ligand-receptor interactions, Ro5-4864 can be classified as an agonist and PK11195 as an antagonist or partial agonist (11). The isoquinoline carboxamides have much greater selectivity for the PBR than for the central benzodiazepine receptor (11). The rat (12), mouse (13), bovine (14), and human (15) PBR cDNAs have recently been cloned. All four cDNAs encode 18-kDa proteins, as predicted from gel electrophoresis analysis of the PBR photolabeled with [^{3}H]PK14105. The proteins are hydrophobic, with hydrophobicity analysis indicating five potential transmembrane regions. At least two other proteins present in the membrane environment, the voltage-dependent anion channel protein and the adenine nucleotide carrier, are associated with the PBR and are perhaps necessary for the constitution of a functional receptor (13, 16, 17). Although Ro5-4864 and PK11195 are able to displace each other from the PBR (11, 12, 18, 19), there is evidence that suggests that the binding domains of the two compounds are not identical (20–23). It has been

ABBREVIATIONS: PBR, peripheral benzodiazepine receptor; Ro5-4864, 4′-chlorodiazepam; PK11195, 1-(2-chlorophenyl)-*N*-methyl-*N*-(1-methylpropyl)-3-isoquinoline carboxamide; PK14105, 1-(2-fluoro-5-nitrophenyl)-*N*-methyl-*N*-(1-methylpropyl)-3-isoquinoline carboxamide; PCR, polymerase chain reaction.



Ro5-4864 PK11195

Fig. 1. Chemical structures of Ro5-4864 and PK11195.

previously suggested that basic residues (24), particularly those in the amino-terminal region of the protein, may be involved in the binding of PK11195 (15). In addition, the systematic replacement of segments of the human receptor with segments of the bovine receptor identified a small region near the carboxyl terminus of the protein as being important for the binding of Ro5-4864 (23).

In an attempt to define regions of the receptor involved in the interaction with PK11195 and Ro5-4864 and to address the question of the topology of the molecule in the membrane, we constructed mutant human PBRs with amino- and carboxyl-terminal deletions and point mutations in potentially accessible cytoplasmic regions of the molecule. The mutant genes were expressed in yeast, a homogeneous cellular environment devoid of endogenous PBR (25), and analyzed in binding experiments using radiolabeled PK11195 and Ro5-4864. These experiments showed that, whereas deletions in the amino-terminal sequence considerably affected the binding of both ligands, the hydrophilic carboxyl-terminal sequence could be deleted with no effect on the binding of either molecule. Furthermore, these experiments allowed us to identify three charged residues, Glu-29, Arg-32, and Lys-39, in the amino-terminal region of the sequence and one hydrophobic residue, Val-154, in the carboxyl-terminal region as being clearly involved in the binding site of Ro5-4864 but not in that of PK11195. The exchange of Met-154 for valine in the bovine PBR resulted in a receptor capable of recognizing Ro5-4864 as well as PK11195, indicating that this residue plays a key role in the recognition of Ro5-4864. These results suggest that the first putative hydrophilic loop and the carboxyl-terminal sequence are close to each other and are exposed to the cytoplasm. They also show at the molecular level that binding sites for Ro5-4864 and PK11195 are overlapping but not identical.

Materials and Methods

Bacteria and yeast strains. *Escherichia coli* strains were TG1 [*hsdS, proAB, lacZX174, (F'pro+, lacZΔM15)*] and S17-1 [*recA, thi, pro, hsdR (RP4-2-Tc::Mu-km::Tn7*] (23). C13ABYS86 (*MATa, ura3, leu2, his3, pra1, prb1, prc1, cps1*) is a multiprotease-deficient strain of *Saccharomyces cerevisiae* obtained from D. Wolf (University, Stuttgart) (26). Bacterial strains were grown in LB medium containing 100 μg/ml ampicillin or in 2YT (1% tryptone, 1% yeast extract, 0.5% NaCl) (27). Yeast strains were grown in complex YP (1% yeast extract, 2% bacto-peptone) or minimal YNB (0.67% yeast nitrogen base) medium supplemented with either 2% glucose (YNBG) or 2% galactose.

Site-directed mutagenesis. All synthetic oligonucleotides were purified by 8% polyacrylamide/7 M urea gel electrophoresis (Amresco, Solon, OH). Mutations in the human receptor gene were obtained either by site-directed mutagenesis or by PCR using mutated oligonucleotide primers. As template for site-directed mutagenesis, we constructed a single-stranded vector (mp19-PBR) containing all of the human PBR gene (15) inserted into M13 mp19. Annealing with the mutagenic oligonucleotides, filling, ligation, and recovery of the mutated strand were performed as recommended by the manufacturer of the mutagenesis kit (Sculptor; Amersham, Paris, France). For amino- or carboxyl-terminal deletions, PCR-directed mutagenesis was performed with mutated oligonucleotide carrying an initiation or termination codon. As typical examples, 1) the deletion mutant Δ157–169 PBR was constructed by PCR using a mutated antisense primer, 5′-cg ggatcccgttaACGCCATACGCAGTAGTTGAGTGT, (Lower case letter represent the nonpriming end containing a BamHI site for further subcloning) and a sense primer corresponding to the coding sequence of the receptor gene, 5′-CTGCAGAAGCCCTCGTGGCACCCGC, and 2) the mutation M154V in the bovine PBR was obtained by site-directed mutagenesis with the mutated phosphorylated oligonucleotide 5′-pGCATGCTCAACTATagagtaTGGCAGGACAAT. All mutated genes were fully sequenced over the entire coding region, using the chain-termination method. The DNA fragment corresponding to the mutated gene was inserted into the expression plasmid pEMR 780 (23) for production of the recombinant receptors, as described (25).

Recombinant receptor expression, subcellular fractionation, and binding of [^{3}H]PK11195 and [^{3}H]Ro5-4864. Yeast transformants were grown for 16 hr in YNBG supplemented with 0.5% casamino acids. Cells were washed and resuspended in YP medium containing galactose and were grown at 30° C overnight. Mitochondria were isolated as described (28) and used for the binding experiments. Saturation experiments with [^{3}H]PK11195 (85.0 Ci/mmol; New England Nuclear) or with [^{3}H]Ro5-4864 (84.0 Ci/mmol; New England Nuclear) were performed at least three times in triplicate with the mitochondrial fraction of the cell-free extracts, as described previously (17, 25). Specific binding was calculated as the difference between the binding in the absence and in the presence of unlabeled PK11195 or Ro5-4864 (both synthesized at Sanofi); nonspecific binding represented <10% of total binding. Data were analyzed using a nonlinear curve-fitting procedure based on the Levenberg-Marquard algorithm (RS/1 statistical software; Bolt Berenek and Newman, Cambridge, MA). The differences between the K_d values determined for the wild-type and mutated receptors were tested by analysis of variance, and changes were considered significant at $p < 0.05$.

Results

Deletion mutants of the human PBR. The PBR is a 18-kDa hydrophobic protein with five putative transmembrane regions located in the outer mitochondrial membrane (Fig. 2). PK11195 and Ro5-4864 bind to the human PBR on possibly overlapping but not identical sites. As a first step in defining the regions of the receptor involved in the interaction with Ro5-4864 and PK11195, we constructed mutant PBRs with amino- and carboxyl-terminal deletions. The mutant genes were expressed in yeast, and the mitochondria were isolated and used in binding experiments. The deletion of the residue 2–20 sequence (Δ2–20 PBR) resulted in a reduction of the binding affinity for both ligands, but the binding of Ro5-4864 was the more affected (Table 1). The more drastic deletion of the residue 15–35 sequence (Δ15–35 PBR) resulted in the complete loss of binding of both ligands. The deletion of the last 11 or 12 amino acids of the receptor at its carboxyl terminus (Δ158–169 PBR and Δ157–169 PBR) did not significantly alter the binding properties of either PK11195 and Ro5-4864. However, the deletion of one extra carboxyl-terminal amino acid (Δ156–169 PBR) abolished the binding of Ro5-4864, with no effect on the binding of PK11195. The simultaneous deletion

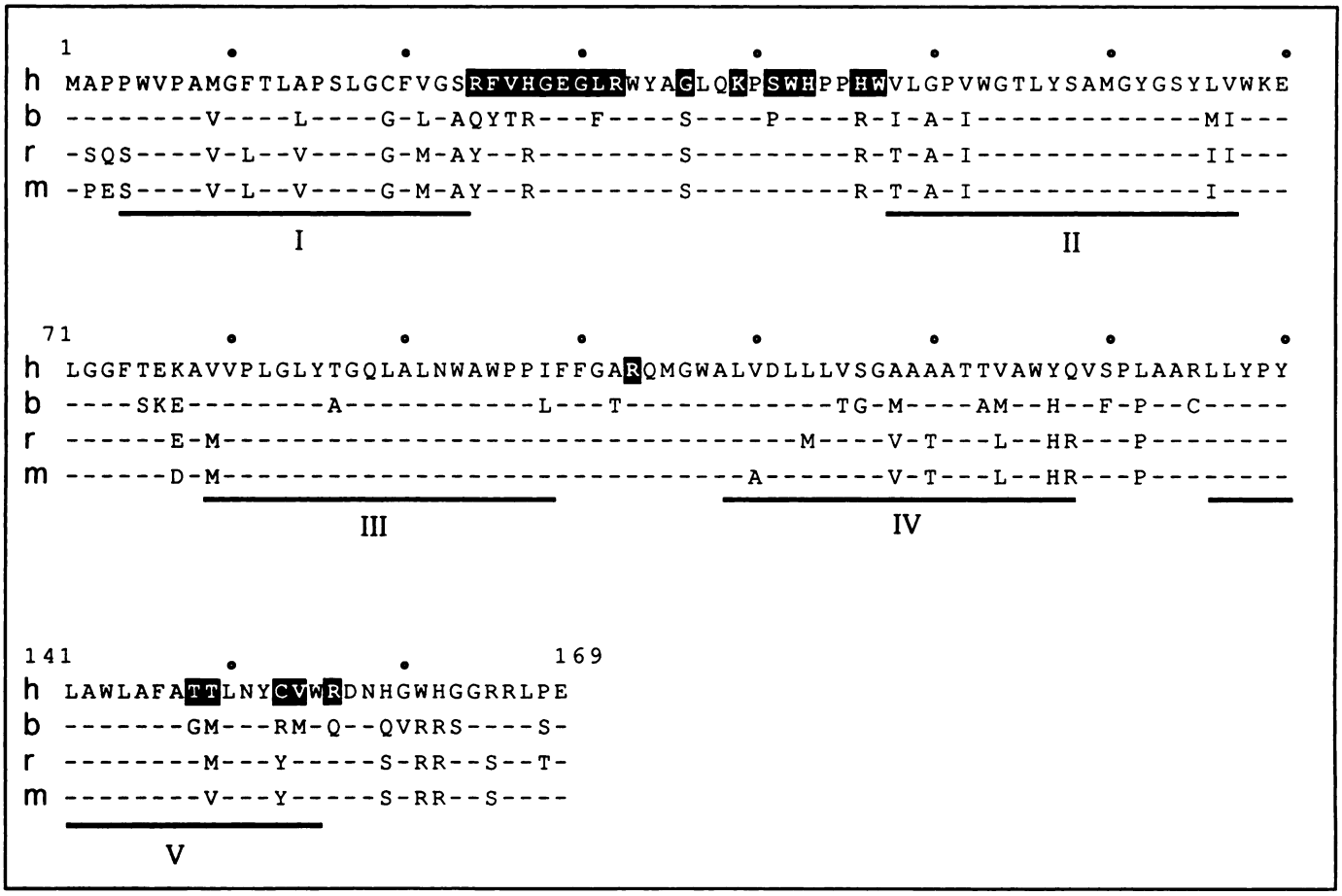


Fig. 2. Alignment of the deduced amino acid sequences of the human (*h*) (15), bovine (*b*) (14), rat (*r*) (12), and mouse (*m*) (13) PBRs. Amino acids are represented using the standard single-letter code; *hyphens* represent conserved residues, compared with the human sequence. Sequences predicted by hydropathic analysis to constitute the five transmembrane regions are *underlined*. *Highlighted amino acids* are those that were mutated as described in the text.

TABLE 1

Affinity of PK11195 and Ro5-4864 for the amino- and carboxyl-terminal sequence-deleted human PBRs

The open boxes represent the PBR, the hatched boxes are the deletions, and the highlighted Roman numerals in the wild-type PBR identify the putative transmembrane regions.

		K_d	
		PK11195	Ro5-4864
		nM	
1 — 169	Wild-type PBR	6 ± 3	9 ± 5
	Δ2–20	25 ± 8[a]	120 ± 45[b]
	Δ15–35	>200[b]	>200[b]
	Δ158–169	10 ± 6	36 ± 4[a]
	Δ157–169	9 ± 3	11 ± 1
	Δ156–169	10 ± 4	>200[b]
	Δ2–20,158–169	>200[b]	>200[b]

[a] Statistical difference from control, $p < 0.05$.
[b] $p < 0.001$.

of both amino- and carboxyl-terminal sequences (Δ2–20,158–169 PBR) completely abolished the binding of both ligands. We confirmed by Western blot analysis that the mutant non-binding proteins Δ15–35 PBR and Δ2–20,158–169 PBR were expressed and present in the yeast mitochondrial membrane (data not shown). The possibility that the deleted proteins had undergone major conformational changes and/or were poorly orientated in the mitochondrial membrane as a consequence of the modifications could not, however, be excluded. Nevertheless, guided by these results, we undertook the construction and analysis of PBRs altered by site-directed mutagenesis.

Neutral point mutations in the human PBR near the amino terminus. It has been suggested (15, 24) that the charged amino acids, Arg-24, Glu-29, Arg-32, and Lys-39, situated in the first putative cytoplasmic loop (see Figs. 2 and 5) may be part of, or close to, the PK11195 binding site. Three of these, Glu-29, Arg-32, and Lys-39, are fully conserved in the four receptors that have been cloned (Fig. 2). Arg-24 is replaced

by glutamine in the bovine PBR sequence and by tyrosine in the rat and mouse sequences. Because the four PBRs bind PK11195 with high affinity, we first targeted the conserved amino acids. Arg-32 and Lys-39 were replaced by glycine or by alanine, as either single or double mutations. Each of the mutated receptors was expressed in yeast and analyzed in binding experiments; the results are shown in Table 2. The replacement of these basic amino acids resulted in a significant reduction in binding for Ro5-4864, whereas the binding of PK11195 was, unexpectedly, unaffected, with the results being comparable for all of the variants tested (Fig. 3B; Table 2). The acidic residue Glu-29 was replaced in a triple-mutant in which the perfectly conserved Gly-28/Glu-29/Gly-30 sequence was replaced by an alanine triplet. Here again, the mutated receptor had the binding characteristics of the wild-type receptor with PK11195, whereas Ro5-4864 binding was drastically decreased (Fig. 3C; Table 2). Taken together, these results suggest that Glu-29, Arg-32, and Lys-39 are involved in the binding of Ro5-4864, whereas, of these three amino acids, only Arg-32 may play at most a minor role in the recognition of PK11195.

Other amino acids in the first cytoplasmic loop that are particularly interesting are the histidines, following reports that they may be involved in the binding of PK11195 (20, 21). The deletion experiments eliminated a possible implication in ligand binding of the two other histidines exclusively present in the human PBR, i.e., His-159 and His-162 (Table 1). The single-mutants H27A and H46A showed binding affinities similar to those of the wild-type receptor (Table 2). His-43 is the only fully conserved histidine in the four cloned receptors; its replacement by alanine resulted also in a PBR with affinities for Ro5-4864 and PK11195 similar to those of the wild-type receptor. Thus, it appears clear that none of these three histidines is implicated in the recognition of either of the two ligands by the human PBR. This conclusion was reinforced by the binding results obtained with the quadruple-mutant W42A,H43A, H46A,W47A (Table 2), results that also showed that the fully conserved Trp-42 and Trp-47 residues are part of neither the PK11195 nor the Ro5-4864 binding sites.

In the second putative cytoplasmic loop, the charged residue Arg-103 was also changed to alanine. The resulting mutant, R103A, was similar to the wild-type receptor when analyzed for binding of PK11195 and Ro5-4864 (Table 2), showing that this charged amino acid was not necessary for recognition of either molecule.

TABLE 2

Affinity of PK11195 and Ro5-4864 for mutated PBRs in which the residue in the human sequence was replaced by alanine or glycine

	K_d	
	PK11195	Ro5-4864
	nM	
Wild-type PBR	6 ± 3	9 ± 5
R32G	12 ± 5	51 ± 10[a]
K39G	6 ± 2	43 ± 12[b]
R32G,K39G	9 ± 3	42 ± 5[a]
R32A,K39A	13 ± 3	75 ± 11[a]
G28A,E29A,G30A	8 ± 2	94 ± 15[a]
H27A	11 ± 2	11 ± 1
H43A	13 ± 1	6 ± 1
H46A	9 ± 1	16 ± 2
W42A,H43A,H46A,W47A	12 ± 3	18 ± 4
R103A	6 ± 1	15 ± 2
R156A	6 ± 2	8 ± 1

[a] Statistical difference from control, $p < 0.001$.
[b] $p < 0.05$.

Human to bovine point mutations near the amino terminus of the PBR. After the identification of Glu-29, Arg-32, and Lys-39 as being implicated in the recognition site of Ro5-4864, and recalling that the bovine receptor cannot be labeled with Ro5-4886 (23), we looked at other candidate residues in the first putative cytoplasmic loop that might be implicated in the differential binding of the benzodiazepine to the human and bovine PBR, namely R24Q, F25Y, V26T, H27R, L31F, G36S, S41P, and H46R. With the histidines having been eliminated from being implicated in binding, we turned our attention to the other amino acids present in the first cytoplasmic region of the human PBR, i.e., Arg-24, Phe-25, Val-26, Leu-31, Gly-36, and Ser-41. These were mutated to the corresponding bovine residues. The results of the binding analysis of the mutated receptors are shown in Table 3. The small variations in K_d values observed suggest that these amino acids are not involved in the binding site of Ro5-4864 or, as expected, in that of PK11195. The mutant V26T exhibited very low specific binding for PK11195 and almost none for Ro5-4864, but Western blot analysis showed that this mutated protein was very poorly expressed in the yeast mitochondria. No firm conclusion can be drawn from results with this mutation until we can express the protein at higher levels.

Point mutations in the carboxyl-terminal region of the human PBR. The carboxyl-terminal region was also analyzed by site-directed mutagenesis. The mutants with shortened carboxyl-terminal sequences suggested that Arg-156 was important for the binding of Ro5-4864 (Table 1). However, when this amino acid was replaced by alanine in the mutant R156A, we observed that the binding affinites for Ro5-4864 and PK11195 were not modified (Table 2). Thus, the results with the deletion mutant Δ156–169 PBR may simply reflect poor insertion of the receptor into the membrane, resulting from the deletion of all of the charged amino acids at the carboxyl-terminal sequence. We recently reported (23) that amino acids between residues 144 and 157 were probably involved in the recognition of Ro5-4864 but not of PK11195. The differences between the human and bovine sequences in this region are T148G, T149M, C153R, and V154M. We therefore constructed two doubly mutated receptors, i.e., T148G,T149M and C153R,V154M. Binding analysis showed that the mutant T148G,T149M conserved the binding of PK11195 and Ro5-4864, whereas the other, C153R,V154M, recognized only PK11195 (Table 3), suggesting that Cys-153 and/or Val-154 are involved in the binding of Ro5-4864. Binding analysis of the single-mutant C153R showed that the binding of both Ro5-4864 and PK11195 was unchanged, immediately indicating the involvement of Val-154. Confirmation of this involvement came from the results with the V154M mutant, which, while maintaining a high affinity for PK11195, was not able to recognize Ro5-4864. These results pinpointed the hydrophobic amino acid Val-154 as a key residue for the recognition of Ro5-4864. It followed that the absence of this residue in the bovine PBR probably accounted for the nonrecognition of Ro5-4864 by the bovine receptor (Fig. 4A). We confirmed the importance of this residue for the binding of Ro5-4864 by constructing a bovine receptor in which the original Met-154 was replaced by valine. When analyzed in binding experiments, the bovine M154V receptor was able to recognize

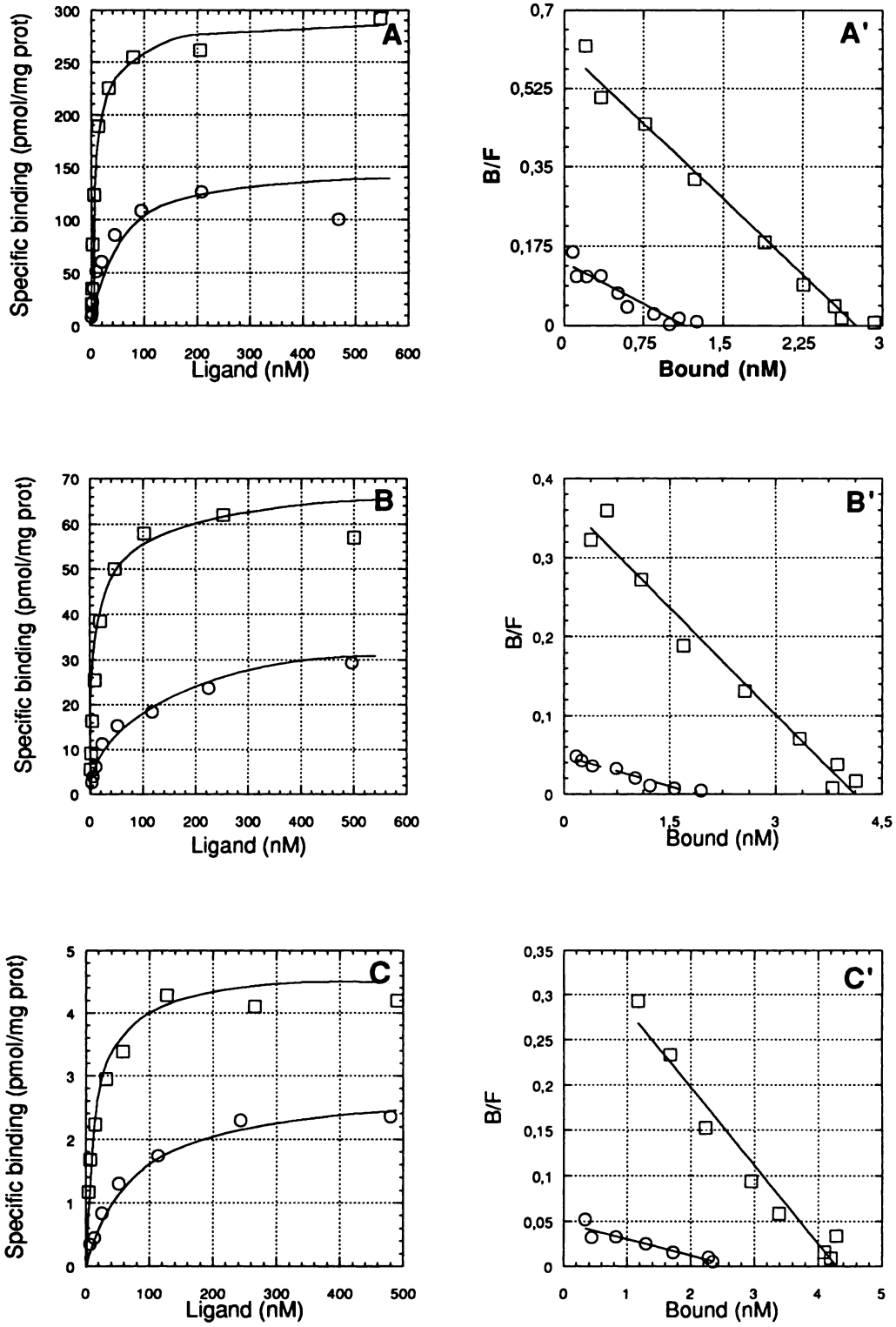


Fig. 3. Analysis of PK11195 and Ro5-4864 binding to wild-type and point-mutated human PBRs. Isolated mitochondria from yeast expressing wild-type human PBR (A), R32G human PBR (B), or E29A human PBR (C) were incubated with [^{3}H]PK11195 (0.6–500 nM) (□) or [^{3}H]Ro5-4864 (0.7–480 nM) (○). Scatchard analysis of PK11195 (□) and Ro5-4864 (○) specific binding to wild-type PBR (A′), R32G human PBR (B′), or E29A human PBR (C′) is also shown. Data are means of triplicate determinations from representative experiments that were repeated at least three times. Data were fitted as described in Materials and Methods.

TABLE 3

Affinity of PK11195 and Ro5-4864 for mutated PBRs in which the residue in the human sequence was replaced by the corresponding residue in the bovine sequence

	K_d	
	PK11195	Ro5-4864
	nM	
Wild-type PBR	6 ± 3	9 ± 5
R24Q	11 ± 2	22 ± 5
F25Y	13 ± 1	14 ± 4
V26T[a]	[b]	>200[c,d]
L31F	16 ± 1	24 ± 2
G36S,S41P	16 ± 2	15 ± 1
T148G,T149M	5 ± 3	11 ± 2
C153R,V154M	2 ± 1	>200[d]
C153R	5 ± 3	15 ± 2
V154M	6 ± 3	>200[d]

[a] The mutant receptor was expressed at a very low level.
[b] The specific binding was insufficient to calculate a K_d.
[c] No specific binding was detected with up to 200 nM Ro5-4864.
[d] Statistical difference from control, $p < 0.001$.

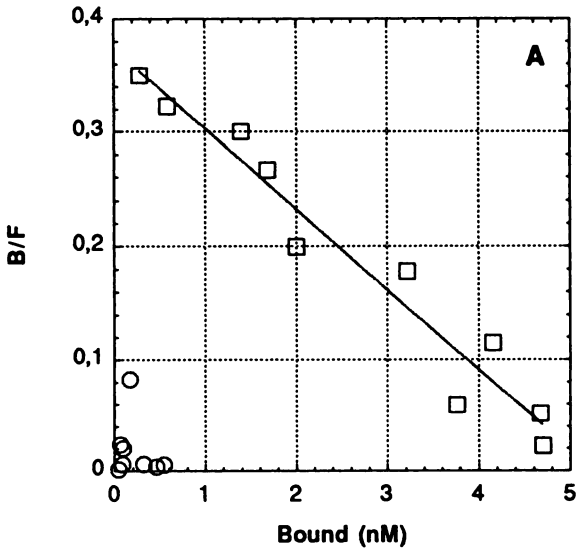
Ro5-4864 as well as PK11195, with K_d values of 8 ± 3 nM and 14 ± 4 nM, respectively (Fig. 4B).

Discussion

To address the question of the orientation of the 18-kDa PBR in the mitochondrial membrane and to define at the molecular level the regions of the receptor involved in the interactions with PK11195 and Ro5-4864, we constructed amino- and carboxyl-terminally deleted receptors, as well as receptors mutated in potentially accessible cytoplasmic regions. The modified genes were expressed in yeast, a microorganism devoid of endogenous PBRs, and analyzed in binding experiments with isolated mitochondria using radiolabeled PK11195 and Ro5-4864 as ligands.

The amino-terminal sequence of the PBR is hydrophobic and resembles a signal peptide but is not cleaved when the protein is incorporated into the mitochondrial membrane (15, 17). The deletion experiments suggested that amino acids near the amino terminus might be involved in the binding sites of Ro5-4864 and PK11195. However, even if a change (Δ2–20 PBR) or a loss (Δ15–30 PBR) of affinity for Ro5-4864 and PK11195 could be attributed to the removal of key amino acids in the recognition sites, major conformational changes in the topology of the receptor resulting from the deletions could not be excluded. In fact, the absence of charged residues and the hydrophobic characteristics of the first 23 amino acids suggested that they may constitute the first transmembrane region (Fig. 2). As a consequence, even though it may not be a typical mitochondrial targeting sequence, this region may be essential for guiding the correct insertion of the rest of molecule into the membrane.

In contrast to the amino-terminal sequence, the carboxyl-terminal sequence of the PBR is highly hydrophilic, and in the proposed models (29, 30) (Fig. 5) it is exposed to the cytoplasmic environment. The experiments involving deletions at the carboxyl terminus of the PBR clearly suggested that the final 12 amino acids are not involved in the recognition of either Ro5-4864 or PK11195. The analysis of the different carboxyl-terminally deleted receptors pointed to Arg-156 as being important for the binding of Ro5-4864 but not for the binding of PK11195. However, when this amino acid was replaced by alanine in the



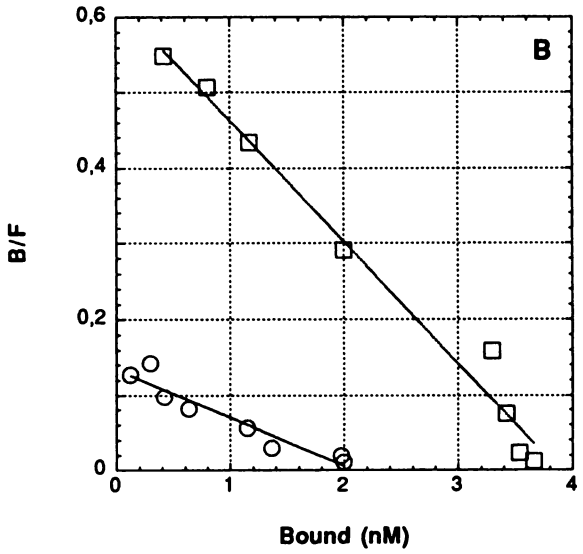


Fig. 4. Scatchard analysis of PK11195 and Ro5-4864 binding to wild-type and point-mutated bovine PBRs. Isolated mitochondria from yeast expressing wild-type bovine PBR (A) or M154V bovine PBR (B) were incubated with [^{3}H]PK11195 (0.6–550 nM) (□) or [^{3}H]Ro5-4864 (0.7–470 nM) (○). Data are means of triplicate determinations from representative experiments. Data were fitted as described in Materials and Methods.

human receptor (R156A), the binding of Ro5-4864 was identical to that displayed by the wild-type receptor. Our results suggest that the correct positioning and stabilization of the last transmembrane region (residues 136–155) require the presence of at least one charged residue at the carboxyl end. Consequently, when Arg-156 is deleted, together with the other last 13 amino acids, the transmembrane domain may not be correctly inserted into the membrane. It should be noted that in the human sequence Arg-156 is followed by another charged residue, Asp-157, and that these two residues are also found in the rat and mouse sequences. In the bovine sequence, Arg-156 is replaced by glutamine, whereas Asp-157 is conserved. Charged dipeptide sequences have been previously described as being important

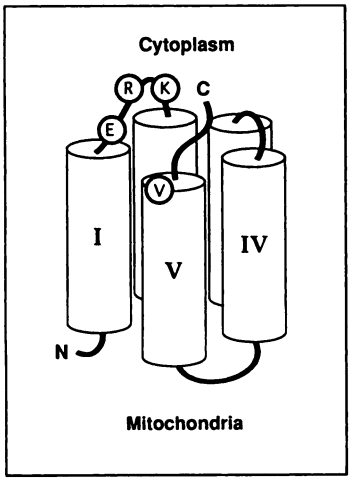


Fig. 5. Simplified topological model of the PBR. The predicted five transmembrane helices are organized in a pore-forming structure (31). The amino-terminal sequence is located in the intermembrane region of the mitochondria, and the carboxyl-terminal sequence is exposed to the cytoplasm. The amino acids in *circles*, Glu-29, Arg-32, and Lys-39 in the first cytoplasmic loop and Val-154 in the carboxyl-terminal region of the fifth transmembrane region, are implicated in the binding site of Ro5-4864.

for orienting and maintaining the transmembrane domains in place (31). The importance of the positioning of the fifth transmembrane region, at least for the binding site of Ro5-4864, is highlighted by our finding that the mutation of one residue in the fifth transmembrane region, Val-154 (V154M), at the interface between the membrane and the cytoplasm in the models based on the hydropathic profile of the human PBR (29, 30) (Figs. 2 and 5), results in the loss of binding of Ro5-4864, with no modification in the binding of PK11195. We confirmed the importance of this residue for the binding of Ro5-4864 by constructing a bovine receptor in which the original Met-154 was replaced by valine. When analyzed in binding experiments, the bovine M154V receptor was able to recognize Ro5-4864 as well as PK11195.

The possible implication of the first putative cytoplasmic loop in the binding of Ro5-4864 and PK11195 was extensively explored by point mutational analysis. The results showed that Arg-32 may play a minor role in the interaction of PK11195 with the receptor and that Glu-29 and Lys-39 are not directly involved in the interaction of the ligand with the PBR. However, these three residues are most likely involved in the interaction of the PBR with Ro5-4864. An indirect effect of the mutations on the Ro5-4864 binding site through conformational changes cannot be excluded but, because the binding of PK11195 was only slightly altered, a major change is unlikely.

The single positively charged residue in the second putative cytoplasmic loop, Arg-103, seems to be outside the binding sites of Ro5-4864 and PK11195. Interestingly, our results also showed that in the human receptor the mutation of the histidines had minor effects on the binding of PK11195 or Ro5-4864. Histidines have been previously implicated in the binding site of PK11195 (20, 21), because the modification of these residues with diethyl pyrocarbonate, a histidine-specific reagent, inhibits up to 70% of PK11195 binding while minimally affecting the affinity or number of Ro5-4864 sites.

Taken together, these results suggest that the first and fifth putative transmembrane regions may be close to each other, to allow the participation of amino acids in the first cytoplasmic loop (Glu-29, Arg-32, and Lys-39) and at the end of the fifth transmembrane region (Val-154) in the binding site of Ro5-4864. Furthermore, the results also suggest that the sequences corresponding to the first loop and the carboxyl-terminal hydrophilic end are exposed to the cytoplasm once the protein is inserted in the mitochondrial membrane. These results are in line with a three-dimensional model that we recently reported, in which the five transmembrane regions are organized in a pore-forming structure (30) (Fig. 5). In this simple model, modification of amino acids located in the external loops, such as the histidines, which are not directly involved in the interaction sites, may inhibit the binding simply by blocking the access of the ligands to the binding site. Because the modifications of the putatively exposed charged amino acids of the PBR did not have any major influence on the binding of PK11195, it is tempting to speculate that PK11195 may interact with hydrophobic residues in the transmembrane regions. New mutants are being constructed to test this possibility. It should also be noted that our results do not exclude other molecular models. For example, to form a functional receptor it may be necessary for several PBR molecules to associate in the membrane in such a way that the amino-terminal and carboxyl-terminal regions of adjacent PBRs are close to each other, to constitute the binding site of Ro5-4864. Another possibility is that there are fewer than five transmembrane regions, with both amino-terminal and carboxyl-terminal regions being on the cytoplasmic side and the receptor lacking a monomeric pore-forming structure. Finally, we do not exclude the possibility that some of the observations described here may be the consequence of altered interactions between the mutated receptor and other mitochondrial membrane proteins, such as the voltage-dependent anion channel protein and the adenine nucleotide carrier, which have been suggested to be part of the functional receptor complex (13, 16, 17). Additional experiments with the deleted and mutated receptors described here, together with others under construction, may help to distinguish between these alternative models. Finally, a better understanding of the Ro5-4864 and PK11195 binding sites on the PBR may contribute to the synthesis of new specific ligands for one or the other binding site, which should lead to a better understanding of the structure and physiological function of this receptor.

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Send reprint requests to: Pascual Ferrara, Sanofi Recherche, Labège Innopole, BP 137, 31676 Labège Cedex, France.