Identification of a Peptide Antagonist to the Peripheral-Type Benzodiazepine Receptor That Inhibits Hormone-Stimulated Leydig Cell Steroid Formation

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ABSTRACT
Peripheral-type benzodiazepine receptor (PBR) is an 18-kDa high-affinity cholesterol and drug ligand-binding protein involved in various cell functions, including cholesterol transport and steroid biosynthesis. To aid our investigation of the biological function of PBR, we have set out to identify functional antagonists. By screening phage display libraries, we have identified peptides that displace the high-affinity PBR benzodiazepine drug ligand, Ro5-4864 (4′-chlorodiazepam). Among these peptides, STPHSTP was the most potent (IC50 = 10 μM). All of the isolated peptides showed a conserved motif STXXXP. The role of these peptides in Leydig cell steroidogenesis was examined using a transducible peptide composed of the TAT domain of human immunodeficiency virus and the peptides under investigation. Synthesized peptides efficiently transduced into MA-10 Leydig cells, and the peptide TAT-STPHSTP inhibited Ro5-4864- and human chorionic gonadotropin-stimulated steroid production in a dose-dependent manner (ED50 = 5 μM). TAT-STPHSTP behaved as a competitive PBR antagonist, which did not affect 22-hydroxycholesterol-supported steroidogenesis. These results yield leads for the development of potent PBR antagonists and indicate that endogenous PBR agonist-receptor interaction is critical for hormone-induced steroidogenesis.

Peripheral-type benzodiazepine receptor (PBR) was originally discovered because it binds the benzodiazepine diazepam with relatively high affinity (Braestrup and Squires, 1977). It was subsequently described as a multicentric complex composed of the 18-kDa receptor protein, the 34-kDa voltage-dependent anion channel protein required for benzodiazepine binding (McEnergy et al., 1992; Garnier et al., 1994), and the 30-kDa adenosine nucleotide carrier (McEnergy et al., 1992) of an as yet unknown function in the complex. Although PBR is present in most tissues examined, it is particularly abundant in steroid-producing tissues where it is found in the outer mitochondrial membrane (Papadopoulos, 1993; Gavish et al., 1999; Casellas et al., 2002). Using high-affinity PBR drug ligands, such as the isoquinoline carboxamide PK 11195 and the benzodiazepine Ro5-4864, it was shown that PBR is involved in the transport of the substrate cholesterol into mitochondria (Papadopoulos, 1993), the rate-determining and hormone-dependent step in steroid biosynthesis. Further studies using PBR-mutant steroidogenic cells (Papadopoulos et al., 1997) and deletion mutation analysis of the 18-kDa PBR protein (Li and Papadopoulos, 1998) demonstrated the determining role of this protein in cholesterol transport. More recently, PBR was shown to be a high-affinity cholesterol-binding protein (Lacapère et al., 2001; Li et al., 2001b). In addition to its function in steroidogenesis, PBR has been also shown to be implicated in mitochondrial respiration (Hirsch et al., 1989), apoptosis (Hirsch et al., 1998; Papadopoulos et al., 1999), and cell proliferation (Miettinen et al., 1995; Hardwick et al., 1999).

In addition to isoquinolines and benzodiazepines (Papadopoulos, 1993; Cassellas et al., 2002), a spectrum of ligands has been identified that bind to PBR with high affinity, such as imidazopyridines, indole derivatives, pyrrolebenzoxazines, and phenoxyphenyl acetamide derivatives (Benavides et al., 1983; Langer and Arbilla, 1988; Romeo et al., 1992; Campiani et al., 1996; Cully et al., 2001). Moreover, porphyrins (Snyder et al., 1987) and the polypeptide DBI (Besman et al., 1989; Papadopoulos et al., 1991a) have been

ABBREVIATIONS: PBR, peripheral-type benzodiazepine receptor; PK 11195, 1-(2-chlorophenyl)-N-methyl-N-(1-methyl-propyl)-3-isoquinoline carboxamide; Ro5-4864, 4′-chlorodiazepam; DBI, diazepam binding inhibitor; PBS, phosphate-buffered saline; TBST, Tris-buffered saline/Tween 20; hCG, human chorionic gonadotropin; ANOVA, analysis of variance; HIV, human immunodeficiency virus.
identified as endogenous PBR ligands. All the above ligands bind to PBR with affinities ranging from nanomolar to high micromolar and stimulate steroid biosynthesis in various cell systems (Papadopoulos, 1993). Despite the large number of PBR ligands developed, there is as yet no PBR antagonist available. Based on the entropy- and enthalpy-driven nature of ligand-receptor interactions, PK 11195 was originally classified as an antagonist and the benzodiazepine Ro5-4864 as an agonist (Le Fur et al., 1983). However, this classification has not been established because, depending on the biological system used, both ligands induce similar effects (Cassellas et al., 2002). The only exception has been the benzodiazepine flunitrazepam, which was found to stimulate basal steroid formation with low potency and partially inhibit hormone-stimulated steroidogenesis (Papadopoulos et al., 1991b). This finding suggested that flunitrazepam acts as a partial agonist in the presence of the hormone-induced endogenous agonist. Thus, the availability of a PBR antagonist would be critical in evaluating the involvement and understanding the function of this receptor in steroidogenesis and other biological systems.

In the present study, we used random 7-mer peptide phage display technology (Scott and Smith, 1990) and isolated mitochondria from the MA-10 mouse Leydig tumor cell line, which express high amounts of PBR, as a screening tool. We report herein the development and characterization of a peptide inhibitor of PBR ligand binding and antagonist of ligand- and hormone-induced Leydig cell steroid formation.

### Materials and Methods

**Materials.** Purified hCG (batch CR-125 of biological potency 11,900 IU/mg) was a gift from the National Institutes of Health. [3H]PK 11195 (specific activity, 86 Ci/mmol), [3H]Ro5-4864 (specific activity, 88 Ci/mmol), and [1,2,6,7-N3H]progesterone (specific activity, 94.1 Ci/mmol) were obtained from PerkinElmer Life Sciences (Boston, MA). PK 11195 and Ro5-4864 were purchased from Sigma/ RBI (Natick, MA). Peptides were synthesized by Bethyl Laboratories Inc. (Montgomery, TX). All other chemicals used were of analytical grade and were obtained from various commercial sources.

**Cells and Mitochondria Preparation.** MA-10 cells were grown in modified Waymouth’s MB752/1 medium containing 15% horse serum (Papapoulos et al., 1990). Mitochondria were isolated as we previously described (Krueger and Papadopoulos, 1990).

**Biopanning.** The Ph.D.-7 Peptide Library Kit (New England Biolabs, Beverly, MA) was used. The library includes 1.28 × 10⁶ possible 7-mer sequences. As protein target, MA-10-isolated mitochondria were used. The selection procedure was performed according to the manufacturer’s instructions. In brief, a microtiter well was coated overnight with 150 μl of 100 μg/ml MA-10 mitochondria in 1× PBS, using 0.1 M NaHCO₃ (pH 8.6), and blocked with a blocking solution containing 0.1 M NaHCO₃ (pH 8.6), 5 mg/ml bovine serum albumin, and 0.02% NaN₃. PBR function on coated mitochondria was determined by liquid scintillation spectrometry.

**Peptide Transduction into Cells.** Twenty-mer TAT-StHSTP and TAT-STHEETP peptides were synthesized so that they contained an NH₂-terminal 11-mer TAT protein transduction domain (single-letter code, YGRKKRRQRRR) followed by two glycine residues (Gius et al., 1999). Peptides were synthesized by Bethyl Laboratories, Transduction experiments were performed as we previously described (Li et al., 2001b). To determine the efficiency of TAT peptide incorporation into the cells, MA-10 cells were cultured overnight on eight-chambered SuperCell Culture Slides (Fisher Scientific, Pittsburgh, PA) at a concentration of approximately 25,000 cells/chamber. Media were replaced 24 h later with fresh media, and cells were treated with various concentrations of Oregon Green 488-labeled peptides for various time periods. After the incubation period, cells were washed with PBS and examined by fluorescent microscopy using an Olympus BX40 fluorescence microscope.

**Protein Measurement.** Proteins were quantified using the dye binding assay of Bradford (1976) with bovine serum albumin as the standard.

**Statistical Analysis.** Statistical analysis was performed by Student’s t test and one-way analysis of variance (ANOVA) using the InStat (v 3.00) package from GraphPad, Inc. (San Diego, CA).

### Results

To identify PBR peptide ligands, we screened a phage library displaying 7-mer random peptides. As protein target, we used isolated mitochondria from MA-10 mouse Leydig tumor cells. The activity of PBR on immobilized/coated mitochondria was tested using the high-affinity diagnostic PBR ligands [3H]PK 11195 and [3H]Ro5-4864. Immobilized mitochondria maintained the ability to bind both [3H]PK 11195 (Kᵣ = 1.4 nM and Bₘₐₓ = 46 pmol/mg of protein) and [3H]Ro5-4864 (Kᵣ = 1.8 nM and Bₘₐₓ = 40 pmol/mg of protein).

In biopanning experiments, PBR-interacting phage peptides were eluted using either PK 11195 or Ro5-4864. After three rounds of panning, 20 individual peptide phage clones from each eluate (40 single phage clones) were selected, amplified, and sequenced. The majority of isolated phages con-
tained a similar sequence from the 7-mer library (ST[H-P][HE][ES][TH]P; Table 1). Examination of the isolated peptide sequences revealed a tripeptide motif, STXXXXP, with the proline located at the carboxyl terminus of the peptide. Sequences identical or similar to the STXXXXP motif occurred in a considerable number of the examined clones. The remaining individual clones that we identified were distinct from the other peptide phage clones but showed identical or similar amino acids in certain positions (Table 1). Similar results were obtained in three independent experiments.

To examine the effect of the phage clones on PBR ligand-binding characteristics, the four most frequent peptides were chosen for synthesis. These peptides were tested as competitors in PBR ligand binding assays using either [3H]PK 11195 or [3H]Ro5-4864 as ligands (Table 2). These displacement studies indicated that STHEETP and STHEETS peptides displaced the radiolabeled PK 11195 ligand with an IC50 of 10 μM. STHEEHP displaced the binding of [3H]PK 11195 with an IC50 of 290 μM, and STPHSTP displaced the radiolabeled PK 11195 ligand with an IC50 of 145 μM. Similar experiments using radiolabeled Ro5-4864 demonstrated that peptides STHEETP and STHEEHP displaced the radioligand in MA-10 cell mitochondria with IC50 values of 170 and 140 μM, respectively. STHEETS displaced the radiolabeled Ro5-4864 ligand with an IC50 > 300 μM. STPHSTP was the most active peptide displacing [3H]Ro5-4864, with an IC50 of 10 μM (Table 2).

The interaction of peptide STPHSTP with PBR was further examined by saturation isotherm experiments performed in the presence of increasing concentrations of radiolabeled Ro5-4864 and 100 μM STPHSTP. Figure 1 shows that in the absence of the peptide, Ro5-4864 binds to MA-10 PBR with a Kd of 1.8 nM and Bmax of 40 pmol/mg of protein. However, in the presence of STPHSTP, Ro5-4864 ligand binding was dramatically reduced, and specific binding was observed only in the presence of higher concentrations of the radioligand.

To examine the biological effect of STPHSTP, we synthesized two 20-mer peptides that consisted of the NH2-terminal TAT protein transduction domain (11 amino acids; Gius et al., 1999), followed by two glycine residues for free bond rotation, and at the carboxyl-terminal, either the 7-mer STPHSTP peptide (TAT-STPHSTP) or the 7-mer STHEETS (TAT-STHEETS) peptide to be used as control. Preliminary experiments using Oregon Green 488-labeled peptides showed that the TAT peptides transduced rapidly into the majority of the cells (data not shown) in agreement with our previous results (Li et al., 2001b). In 30 min, most of the cells were labeled.

Ro5-4864 (1 μM) increased progesterone formation by MA-10 Leydig cells by 3.5-fold (Fig. 2), in agreement with our previous studies (Papadopoulos et al., 1990; Culty et al., 2001). Increasing concentrations of TAT-STPHSTP, but not TAT-STHEETS, inhibited the Ro5-4864-induced steroid formation with an ED50 of 5 μM (Fig. 2). Complete inhibition was achieved in the presence of 100 μM TAT-STPHSTP. Figure 2B shows that 70 μM TAT-STPHSTP inhibited only 50% of the progesterone synthesized in response to 10 μM Ro5-4864, suggesting that TAT-STPHSTP was acting as a competitive PBR antagonist.

In addition, TAT-STPHSTP was found to inhibit in a dose-

### TABLE 1

<table>
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<th>Amino Acid Sequence</th>
<th>Frequency</th>
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### TABLE 2

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<th>IC50 Ro5-4864</th>
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</tr>
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<td>STHEEHP</td>
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</tr>
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<td>STPHSTP</td>
<td>145 μM</td>
<td>10 μM</td>
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**Fig. 1.** Effect of STPHSTP on [3H]Ro5-4864 binding to MA-10 mitochondria. Binding experiments were performed in the presence (open circles) or absence (closed circles) of 100 μM STPHSTP. Saturation isotherms were analyzed as described under Materials and Methods. All data are expressed as the means of triplicate assays where the S.D. was <15% in all replicates.
dependent manner the MA-10 mouse Leydig tumor cell hCG-stimulated steroidogenesis with an ED_{50} of 5 \mu M (Fig. 3A). Concentrations as low as 1 \mu M TAT-STPHSTP induced a significant inhibition of hCG-stimulated steroid formation (p < 0.01). Maximal inhibition achieved with the peptide was 70% of hCG-stimulated steroid production. Treatment with increasing concentrations of TAT-STHEETS did not affect hormone-induced progesterone synthesis. To examine whether this peptide had any effect on the steroidogenic pathway subsequent to the transport of the substrate cholesterol into the mitochondria, MA-10 Leydig cells were pretreated with increasing concentrations of TAT-STPHSTP for 30 min, and at the end of the incubation, the cells were washed and further incubated with the cholesterol derivative 22R-hydroxycholesterol, which diffuses freely across the membranes to reach the cytochrome P450 side-chain cleavage enzyme responsible for cleaving cholesterol into pregnenolone. Figure 3B shows that the responses of the cells treated with either the bioactive TAT-STPHSTP or the control TAT-STHEETS peptide to 22R-hydroxycholesterol were identical.

**Discussion**

In this investigation a 7-mer peptide phage display library was selected against mitochondrial PBR. As protein target for the displacement procedure, we used isolated mitochondria from MA-10 mouse tumor Leydig cells, which contain high levels of PBR located in the outer mitochondrial membrane (Papadopoulos et al., 1990). To eliminate the interaction of phage library with other mitochondrial proteins, specifically bound phages were eluted with an excess of the two diagnostic high-affinity PBR ligands, the isoquinoline PK 11195 and the benzodiazepine Ro5-4864.

Isolated peptide phage clones were found to share common features in their amino acid composition. Serine, threonine, histidine, glutamic acid, and proline appeared frequently. The predominant amino acid sequences found were STHEETP and STHEETS, eluted with PK 11195, and STHEEHP and STPHSTP, eluted with Ro5-4864. The ability of these peptides to displace radiolabeled PK 11195 and Ro5-4864 was further examined to define the affinity of the peptides for PBR. STHEETP, STHEEHP, and STPHSTP, which share the STXXXXXXXX motif, displaced [3H]Ro5-4864 in competition experiments. STPHSTP exhibited the best affinity, displacing radiolabeled Ro5-4864 with an IC_{50} of 10 \mu M. STPHSTP also displaced radiolabeled PK 11195, but with an IC_{50} of 145 \mu M. STHEEHP was the only other peptide that showed some affinity for PBR, exhibiting IC_{50} of 140 and 290 \mu M for Ro5-4864 and PK 11195, respectively. Neither of the other peptides could compete for [3H]PK 11195 binding. It is interesting that in competition studies, the STHEETS peptide, which was eluted with PK 11195, could not displace either of the two PBR ligands tested. STHEETS differs from the other peptides in the seventh amino acid, which is a serine, whereas in the other peptides, there is a proline, suggesting that proline is essential for the function of these peptides. High concentrations of STPHSTP inhibited Ro5-4864 binding, and higher concentrations of the radiolabeled benzodiazepine were required to obtain specific binding. It should be noted that we were unable to calculate the affinity and capacity of the receptor under these conditions. Interestingly, STPHSTP showed 10-fold higher affinity for Ro5-4864 compared with PK 11195, indicating that the peptide can discriminate between the two PBR drug ligands. These data also suggest that either the isoquinoline and benzodiazepine ligands bind on distinct but overlapping binding sites, or there are additional, although distinct, requirements for PK 11195 and Ro5-4864 binding to the 18-kDa PBR protein. The recent identification, using the yeast two-hybrid system, of two proteins that interact with PBR (Gallegue et al., 1999; Li et al., 2001a) suggests that PBR ligand binding may be subject to requirements specific to its microenvironment, which might also reflect cell- and tissue-specific biological functions.

Leydig cells contain high levels of mitochondrial PBR involved in the transport of cholesterol into mitochondria (Papadopoulos, 1993), the rate-determining step in steroid biosynthesis. To examine the biological role of STPHSTP in a physiological function mediated by PBR, i.e., PBR drug ligand- and hormone-stimulated steroid biosynthesis (Papado-
poulos et al., 1990), we introduced the synthetic peptide into Leydig cells. To achieve rapid transduction of high levels of the cholesterol recognition/interaction amino acid consensus (CRAC) domain of PBR into the MA-10 Leydig cells, we used the TAT domain of the HIV TAT protein. This approach, based on the finding that chemical crosslinking a 36-amino acid domain of TAT to heterologous proteins conferred the ability to transduce these proteins into the cells (Fawell et al., 1994), was recently applied to various proteins (Nagahara et al., 1998; Vocero-Akbani et al., 1999), including the carboxyl-terminal cholesterol-binding domain of PBR in MA-10 Leydig cells (Li et al., 2001b). Treatment of cells with the peptide containing the protein transduction domain from HIV TAT protein results in a rapid transduction into ~100% of cells in a given population in a receptorless fashion (Nagahara et al., 1998; Vocero-Akbani et al., 1999; Li et al., 2001b). In addition, because of its concentration dependence, TAT-mediated transduction results in a near-equimolar intracellular concentration of the transduced protein from cell to cell in the population. In this study, we synthesized 20-mer peptides that consisted of an NH2-terminal TAT protein transduction domain (11 amino acids; Gius et al., 1999), followed by two glycine residues for free bond rotation and the COOH-terminal 7-mer STPHSTP or the inactive STHEETS peptides.

TAT-STPHSTP, but not TAT-STHEETS, transduced into MA-10 Leydig cells inhibited in a dose-dependent manner both Ro5-4864 and hCG-induced progesterone production by the cells with identical ED50 values, 5 μM. It should be noted that this ED50 is close to the IC50 (10 μM) of the peptide observed in Ro5-4864 ligand displacement studies. The finding that TAT-STPHSTP inhibited the steroid formation stimulated by 10 μM Ro5-4864 with an ED50 of 100 μM suggested that this peptide is a competitive antagonist of PBR. Interestingly, the peptide did not completely inhibit hormone-induced steroid formation, suggesting that other, PBR-independent, mechanisms are involved in hormone-regulated cholesterol transport and steroid formation. TAT-STPHSTP did not affect 22R-hydrocholesterol-supported steroidogenesis, suggesting that the peptide did not inhibit the steroidogenic enzymes responsible for cholesterol metabolism into the final steroid products.

Although the antagonistic effect of TAT-STPHSTP on Ro5-4864-stimulated steroid synthesis can be explained by the direct inhibition of the drug binding by the peptide, inhibition of hormone-activated steroid synthesis by TAT-STPHSTP is probably due to the inhibition of the binding of an endogenous ligand to the receptor. Two such ligands have been reported: protoporphyrin IX (Snyder et al., 1987) and DBI (Besman et al., 1989; Papadopoulos et al., 1991a). Protoporphyrin IX bound to Leydig cell PBR with low affinity, at high micromolar concentrations (V. Papadopoulos, unpublished data). In contrast, DBI displaced the radiolabeled Ro5-4864 and stimulated mitochondrial pregnenolone formation with IC50 and ED50 ranging between 0.1 and 1 μM (Papadopoulos et al., 1991a, 1992). In addition, the role of DBI in hormone-stimulated steroidogenesis was shown in various steroidogenic cell types, including MA-10 cells (Besman et al., 1989; Papadopoulos et al., 1991a, 1992), where depletion of DBI levels resulted in inhibition of hCG-stimulated steroid production (Boujrad et al., 1993).

The presence of an amino acid sequence able to inhibit PBR drug ligand binding and function in MA-10 Leydig cells suggested that endogenous proteins may exist containing such a sequence and exerting PBR-antagonistic function. Efforts to isolate a nucleotide sequence from MA-10 and rat testis cDNA libraries encoding for a protein containing the STPHSTP amino acid sequence failed. Screening of the SWISS-PROT and PIR databases for proteins containing the consensus motif STXXXXP identified numerous proteins. A more restricted search using the motif ST[PH][HE][XX][P] identified 69 known and hypothetical proteins, including human retinal-binding protein, tumor suppressor protein MN1, mitotic checkpoint serine/threonine kinase BUB1, and the nucleoporin-like protein RIP. A more restricted search using the motif ST[PH][HE][XX][P] identified only one gene product, the yeast SIS2 protein (halotolerance protein HAL3), proposed to stimulate the expression of certain genes that are periodically expressed during late G1 phase, interact with the serine/threonine protein phosphatase PPZ1, and act as an inhibitory subunit of PPZ1 (di Como et al., 1995; SWISS-PROT).

PBR also has been involved in the regulation of cell proliferation, where high-affinity PBR ligands have been shown at micromolar concentrations to inhibit cell proliferation (Wang et al., 1984; Bruce et al., 1991; Hardwick et al., 1999; Maaser et al., 2001), probably via programmed cell death (Hirsch et al., 1998; Maaser et al., 2001). Studies using the TAT-STPHSTP peptide on the MDA-MB-231 aggressive human breast cancer cell line showed that the peptide failed to block the inhibitory effect of micromolar concentrations of Ro5-4864 on cell proliferation (data not shown). These results suggest that either this function of micromolar concentrations of high-nanomolar-affinity PBR drug ligands is not related to PBR, as previously suggested by Zisterer et al. (1998) and Fennell et al. (2001), or the concentration of the antagonist needed to block the effect of micromolar concentrations of Ro5-4864 is too high to be reached with the culture conditions used.

In conclusion, we have identified a family of specific peptide antagonists for the mitochondrial PBR from the 7-mer peptide phage display library by selection on isolated mitochondria from MA-10 Leydig cells. The active peptides share the motif STXXXXP. STPHSTP was the most potent peptide, displacing high-affinity PBR drug ligands and blocking a PBR-mediated biological function. This peptide might help elucidate the role of PBR in other cell functions, both in vitro and in vivo. The sequence of STPHSTP, and other peptides containing the motif STXXXXP, might serve as leads for the development of potent PBR antagonists.

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