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Met909 Plays a Key Role in the Activation of the Progesterone Receptor and Also in the High Potency of 13-Ethyl Progestins

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ABSTRACT

Many progestins have been developed for use in contraception, menopausal hormone therapy, and treatment of gynecological diseases. They are derived from either progesterone or testosterone, and they act by binding to the progesterone receptor (PR), a hormone-inducible transcription factor belonging to the nuclear receptor superfamily. Unlike mineralocorticoid, glucocorticoid, and androgen receptors, the steroid-receptor contacts that trigger the switch of the ligand-binding domain from an inactive to an active conformation have not yet been identified for the PR. With this aim, we solved the crystal structure of the ligand-binding domain of the human PR complexed with levonorgestrel, a potent testosterone-derived progestin characterized by a 13-ethyl substituent. Via mutagenesis analysis and functional studies, we identified Met909 of the helix 12 as the key residue for PR activation by both testosterone- and progesterone-derived progestins with a 13-methyl or a 13-ethyl substituent. We also showed that Asn719 contributes to PR activation by testosterone-derived progestins only, and that Met759 and Met909 are responsible for the high potency of 19-norprogestins and of 13-ethyl progestins, respectively. Our findings provide a structural guideline for the rational synthesis of potent PR agonist and antagonist ligands that could have therapeutic uses in women's health.

The physiological effects of progesterone are mediated by the progesterone receptor (PR). This receptor belongs to the type-1 nuclear receptor subfamily, which includes the androgen receptor (AR), estrogen receptor, glucocorticoid receptor (GR), and mineralocorticoid receptor (MR). Like other members of the family, the PR contains three main functional domains (Tsai and O'Malley, 1994; Mangelsdorf et al., 1995; Lu et al., 2006). The N-terminal domain of the PRA and PRB isoforms harbors one and two activation functions, respectively. The centrally located DNA-binding domain is involved in DNA binding and in receptor dimerization. The C-terminal domain, or ligand-binding domain (LBD), is involved in ligand binding and transcriptional activation.

PR is a hormone-inducible transcription factor activated by a multistep mechanism. Binding of agonist molecules to PR induces a major conformational change within the LBD that is thought to promote receptor dimerization, and its interaction with DNA at specific response elements located in the regulatory regions of target genes. The agonist-induced conformational change also promotes the recruitment of transcriptional coactivators and the ordered assembly of multiprotein complexes with chromatin-modifying activities (Tsai and O'Malley, 1994; Georgiakaki et al., 2006).

Crystallographic and in vitro studies of numerous nuclear receptors have suggested that the LBD, surrounding the ligand-binding cavity, is rather dynamic in the absence of ligand (Nagy and Schwabe, 2004). Binding a ligand compacts the LBD by establishing many polar and hydrophobic contacts. Some of these contacts are involved only in the stability of the ligand receptor complex, but some others are required to stabilize the LBD in its active conformation. They may do this in two ways. First, the ligand itself may make direct contact with residues of the carboxyl-terminal helix 12, thereby promoting its active conformation. Second, the ligand may stabilize the lower part of the LBD, so that the helix 12 tends to adopt its active position. The crystal struc-

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ABBREVIATIONS: AR, androgen receptor; GR, glucocorticoid receptor; MR, mineralocorticoid receptor; PR, progesterone receptor; LBD, ligand-binding domain; 18OVP, 18-oxo-18-vinylprogesterone (18-vinyl-4-pregnen-3,18,20-trione); GST, glutathione transferase; TENG, Tris-HCl/EDTA/NaCl/*n*-octyl-β-glucoside/glycerol; HNG, HEPES/*n*-octyl-β-glucoside/glycerol; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); HEK, human embryonic kidney; PDB, Protein Data Bank.

tures of the LBD of MR, GR, and AR complexed with an agonist ligand have been solved, and the contacts involved in activating these receptors have been identified (Matias et al., 2000; Bledsoe et al., 2002, 2005). The crystal structures of the human PRLBD associated with various progestins have also been solved (Williams and Sigler, 1998; Madauss et al., 2004; Zhang et al., 2005). Nevertheless, the contact(s) between progestins and the ligand-binding cavity that are required to trigger PR activation remained to be identified, as well as the structural elements that modulate progestin activity.

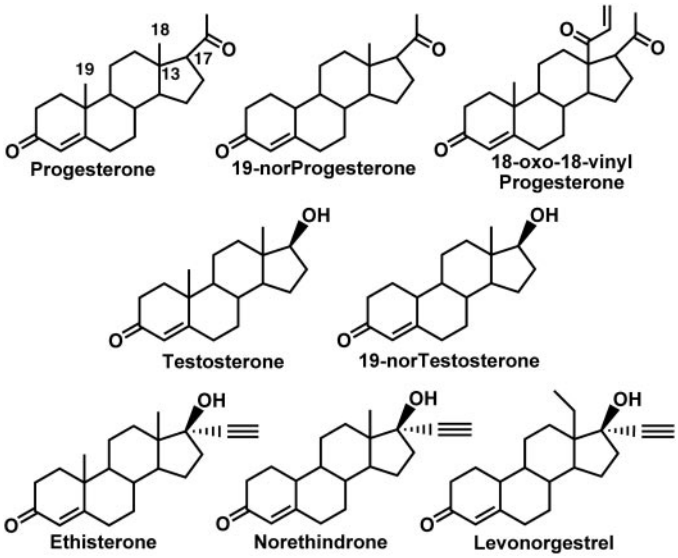

Fig. 1. Structural formula of progestins.

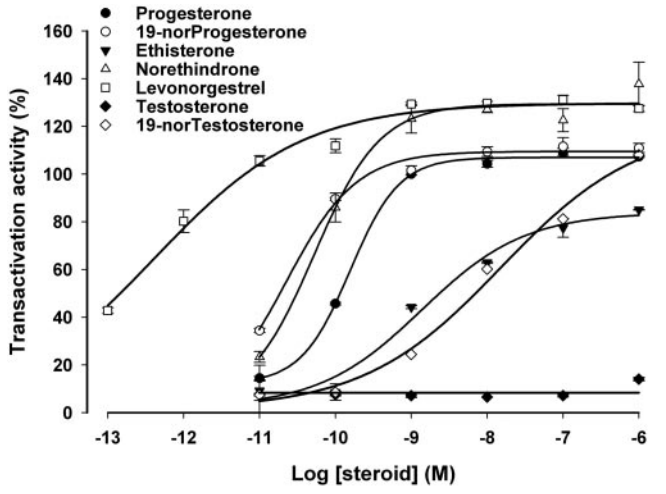

Fig. 2. Effect of ligands on PR trans-activation activity. HEK 293T cells transiently expressing the human PR were incubated for 16 h with increasing concentrations (10^{-13} to 10^{-6} M) of ligands. The PR trans-activation activities were determined from the luciferase activity normalized for β-galactosidase activity. Results are expressed as a percentage of PR activity in response to 10^{-9} M progesterone. Values are mean ± S.E.M. of three separate experiments. A theoretical curve in SigmaPlot has been used to fit the data.

Many progestins have been developed for contraception, menopausal hormone therapy, and treatment of gynecological diseases (Schindler et al., 2003; Stanczyk, 2003; Sitruk-Ware, 2004, 2007). They are derived either from testosterone, and are characterized by a 17β-hydroxyl function, or from progesterone, and display a 17-methyl ketone function. It has been reported that the presence of a 13-ethyl instead of a 13-methyl group increases the affinity of both testosterone- and progesterone-derived progestins (Tuba et al., 2000). To analyze the accommodation mode of progestins harboring a 13-ethyl group, we solved the structure of the PRLBD complexed with levonorgestrel, a potent progestin derived from testosterone and characterized by a 13-ethyl group. By comparing this structure with that of PRLBD complexed with 13-methyl progestins derived from testosterone or progesterone (Williams and Sigler, 1998; Madauss et al., 2004) and using a mutagenesis approach, we identified the contacts involved in PR activation by progestins, and those that modulate their efficiency.

Materials and Methods

Chemicals. Levonorgestrel (13β-ethyl-17α-ethynyl-17β-hydroxy-18,19-dinor-4-androsten-3-one) was from HRA Pharma (Paris, France). 18-Oxo-18-vinylprogesterone (18-vinyl-4-pregnen-3,18,20-trione; 18OVP) was a gift from A. Marquet (Paris, France). Ethisterone (17α-ethynyl-17β-hydroxy-4-androsten-3-one), norethindrone (17α-ethynyl-17β-hydroxy-19-nor-4-androsten-3-one), progesterone (4-pregnene-3,20-dione), 19-norprogesterone (19-nor-4-pregnene-3,20-dione), testosterone (17β-hydroxy-4-androsten-3-one), 19-nor-testosterone (17β-hydroxy-19-nor-4-androsten-3-one), and all other products were purchased from Sigma-Aldrich (St. Louis, MO).

Expression Vectors. The human PR expression vector pchPR was constructed from the pSG5hPRB vector (Georgiakaki et al., 2006). The human PR coding sequence was digested by EcoRI and BglII and subcloned into pcDNA3 vector. The various vectors of mutant PRs ($pchPR_{N719A}$, $pchPR_{M759A}$, or $pchPR_{M909A}$) were obtained from pchPR using the site-directed mutagenesis procedure (Stratagene, Amsterdam, The Netherlands). The plasmid pcβgal, which contains the β-galactosidase sequence, was used to standardize transfection experiments (Fagart et al., 2005). The reporter vector GRE2Luc was a gift from A. Biola-Vidammente and M. Pallardy (Châtenay-Malabry, France). The sequence of the PRLBD (675–933) was amplified by polymerase chain reaction from the pSG5hPRB vector using the following primers: 5′- GCGTGGATCCGGTCAAGACATACAGTTGATTCC-3′ and 5′- CGCGCCTCGAGGATGACATTCACTTTTTATGAAAGAGAAGG-3′. The BamHI-XhoI digested product was cloned into the pGexKG vector (gift from S. Jalaguier) to produce the pGexhPRLBD vector encoding for the fusion protein between GST and PRLBD. The vectors pGEX2TK, encoding glutathione transferase (GST) or GST fused to 716–741 fragment of TIF1α (GST-TIF1α), were provided by V. Cavaillès (Montpellier, France).

Protein Expression and Purification. Fermentation using the BL21 CodonPlus (DE3) RIL strain from Stratagene transformed

TABLE 1
Half-maximally effective concentrations (10^{-11} M) of progestins for PR, PR_{N719A}, and PR_{M759A}
The ED_{50} ± S.E.M. values were calculated by using a Sigma Plot fitting program.

	Progesterone	19-Norprogesterone	Ethisterone	Norethindrone	Levonorgestrel
PR	13.3 ± 1.5	2.2 ± 0.1	86 ± 15	5.5 ± 0.3	0.016 ± 0.005
PR_{N719A}	790 ± 150	14 ± 1			
PR_{M759A}	6.8 ± 1.1	138 ± 2	22 ± 8	190 ± 31	0.39 ± 0.08

with the pGexhPRLBD vector was carried out in the presence of 50 μM levonorgestrel. Expression was induced by incubating with 200 μM isopropyl-β-D-thiogalactoside for 16 h at 15°C. After centrifuging, the bacteria were disrupted by sonication in TENG buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 10% glycerol, and 0.1% *n*-octyl-β-glucoside) supplemented with 50 μM levonorgestrel. The lysate was clarified and loaded onto a GSTrap column (GE Healthcare, Saclay, France). The fusion protein was eluted with 15 mM reduced glutathione in the TENG buffer. After diluting the eluate to a protein concentration of 1 mg/ml, the fusion protein was cleaved by exposing to thrombin protease (100 units/mg of fusion protein) overnight at 4°C. The protein mixture was diluted a further 5-fold in a HNG buffer (10 mM HEPES pH 6.8, 10% glycerol, 0.1% *n*-octyl-β-glucoside) supplemented with 50 μM levonorgestrel, loaded onto a sulfoxide column (SP XL; GE Healthcare, Les Ulis, France), and eluted with a 0-to-500-mM NaCl gradient in the HNG buffer. The fractions containing the LBD were pooled and concentrated to a protein concentration of 4 mg/ml.

Crystallization and Structure Determination. Crystals were grown over 3 weeks at room temperature in hanging drops containing 1 μl of protein solution and 1 μl of well buffer (50 mM PIPES, pH 6.5, 8% PEG3350, 300 mM $MgSO_4$, and 10% glycerol). Diffraction data were collected to a 2.26-Å resolution on the FIP-BM30A beamline at the European Synchrotron Radiation Facility (ESRF, Grenoble, France) using a MarCCD detector. The data set was integrated and scaled using XDS (Kabsch, 1993). The structure was solved by molecular replacement, using Phaser (Storoni et al., 2004) with the coordinates of the PRLBD associated with norethindrone (PDB ID: 1SQN) as the search model. From molecular replacement, several rounds of manual rebuilding using the Sigma A-weighted 2 F_o-F_c electron density maps, followed by simulated annealing and individual isotropic B factor refinements were performed using CNS (Brünger et al.,1998). Solvent molecules were located in a F_o-F_c map contoured at 2σ. The final model was validated with PROCHECK (Laskowski et al., 1993). All structural figures were produced using DINO (http://www.dino3d.org).

Cell Culture and Trans-Activation Assays. HEK 293T cells were cultured in high-glucose Dulbecco's minimal essential medium (Invitrogen, Cergy Pontoise, France), 25 mM HEPES, 100 IU/ml penicillin, and 100 μg/ml streptomycin, supplemented with 10% heat-inactivated fetal calf serum at 37°C in a humidified atmosphere and with 5% CO_2. Thirty hours before transfection, the cells were cultured in the same medium supplemented with 10% charcoal-treated fetal calf serum. Transfections were carried out using the calcium phosphate precipitation method. Cells were transfected with 0.4 μg of the receptor expression vector (pchPR, $pchPR_{N719A}$, $pchPR_{M759A}$, or $pchPR_{M909A}$), 7 μg of GRE2Luc, and 1 μg of pcβgal. Sixteen hours after transfection, the cells were replated in 12-well plates. After 6 h, the steroids were added and the cells were incubated for 16 h. The cell extracts were assayed for luciferase and β-galactosidase activities (Herbomel et al., 1984; de Wet et al., 1987). To standardize the transfection efficiency, the relative light units obtained in the luciferase assay were divided by the optical density obtained in the β-galactosidase assay.

Binding Studies. HEK 293T cells were cultured and transfected with a receptor expression vector (pchPR or $pchPR_{M909A}$) as described above. Sixteen hours after transfection, the cells were replated in 12-well plates. After 24 h, the cells were incubated at 20°C for 30 min with 10^{-9} M [^{3}H]progesterone with or without 10^{-6} M progesterone or levonorgestrel. Cells were rinsed rapidly with Dulbecco's minimal essential medium, and then with PBS. The radioactivity was counted after ethanol extraction.

Coupled Cell-Free Transcription and Translation. [^{35}S]-labeled wild type and mutant PRs were synthesized in vitro using the T7-coupled rabbit reticulocyte lysate system (Promega, Charbonnières, France). They were incubated with 10^{-7} M progestin for 10 min at 20°C, and then with increasing concentrations of trypsin for 10 min at 20°C. The mixtures were boiled for 5 min before being loaded immediately onto 12.5% SDS-polyacrylamide gels, and subjected to electrophoresis. The gels were then fixed for 30 min in methanol/acetic acid/distilled water (30:10:60), treated with Amplify, dried, and autoradiographed at −80°C overnight.

GST Pull-Down Assays. GST and GST-TIF1α (Thénot et al., 1997) were expressed as described previously (Hellal-Levy et al., 2000). A 1-ml aliquot of the crude bacterial extract containing the GST fusion protein was adsorbed for 30 min at 4°C on 25 μl of glutathione-A Sepharose beads. The beads were then washed three times with 0.5% Nonidet P-40, 1 mM EDTA, 20 mM Tris-HCl, and 100 mM NaCl, ph 8.0, containing protease inhibitors. The PR and PR_{M909A} were translated with ^{35}S-labeled methionine in rabbit reticulocyte lysate following the manufacturer's instructions. The receptors expressed in vitro were incubated without (ethanol: no hormone) or with 1 μM levonorgestrel or 18OVP for 10 min at 20°C and then incubated with the fusion proteins loaded onto glutathione-Sepharose beads for 15 min at 20°C. The beads were then washed, resuspended in 20 μl of loading buffer, boiled for 5 min, and analyzed by SDS-polyacrylamide gel electrophoresis. Signals were amplified with Amplify, and gels were dried and autoradiographed at −80°C overnight.

Results

Progestin Potency. Levonorgestrel is a 18,19-dinorsteroid characterized by having 13-ethyl, 17α-ethynyl, and 17β-hydroxyl groups (Fig. 1). We wanted to identify the levonorgestrel substituent(s) responsible for its high potency, so we analyzed the capacity of this steroid to activate the transiently expressed human PR and compared its potency with that of various ligands selected for their chemical structures (Fig. 1). Trans-activation experiments showed that testosterone is nearly unable to activate PR. In contrast, ethisterone, characterized by a 17α-ethynyl group, is able to activate PR (Fig. 2, Table 1). Progesterone is more potent than ethisterone (ED_{50}, 13.3 versus 86 $\times$ 10^{-11} M), indicating that the 17-methylketone function confers a higher potency than the 17α-ethynyl group together with the 17β-hydroxyl group. It is noteworthy that 19-norprogesterone,

TABLE 2
Data collection and refinement statistics

Data collection	PR - Levonorgestrel[a]
Space group	$P2_1$
Cell dimensions	
a, *b*, *c* (Å)	58.87, 65.31, 70.62
α, β, γ (°)	90, 96, 90
Resolution (Å)	47.83–2.26 (2.30–2.26)[b]
R_{merge}	6.7 (31.9)[b]
$I / \sigma I$	11.1 (2.33)[b]
Completeness (%)	80.9 (73.5)[b]
Redundancy	2.76 (2.0)[b]
Refinement	
Resolution (Å)	47.83–2.26
No. reflections	20345
R_{work} / R_{free}	23.0/28.4
No. atoms	
Protein	3967
Ligand/ion	46
Water	352
B-factors	
Protein	43.6
Ligand/ion	40.7
Water	36.7
R.M.S deviations	
Bond lengths (Å)	0.006
Bond angles (°)	1.2

[a] One crystal was used to collect the data.
[b] The highest resolution shell is shown in parentheses.

norethindrone, and 19-nortestosterone seemed to be more potent than progesterone, ethisterone, and testosterone, the analogous molecules with a 19-methyl group (Fig. 2, Table 1). These findings indicate that the absence of the 19-methyl group greatly enhances progestin potency, regardless of the nature of their 17-substituents. Levonorgestrel was the most potent of the progestins tested (ED_{50}, 0.016×10^{-11} M). The fact that levonorgestrel and norethindrone are different only in their C13 substituents suggests that it is the ethyl group that allows levonorgestrel to establish additional stabilizing contacts with the PRLBD.

Crystal Structure of the PRLBD in Complex with Levonorgestrel. To find out how levonorgestrel is accommodated within the ligand-binding cavity of PR, we solved the crystal structure of the PRLBD complexed with levonorgestrel. The PRLBD was expressed as a fusion protein with GST in the presence of a high concentration of levonorgestrel and then purified according to the protocol described previously (Fagart et al., 2005). The structure was solved by molecular replacement and was then refined to 2.26 Å resolution (Table 2). The LBD contains 11 α-helices (H1, H3-H12) and two short β-sheets, organized into three layers. Within the ligand-binding cavity, the Gln725 and the Arg766 residues anchor the C3 ketone function via hydrogen bonds and make contacts with a water molecule. The Asn719 residue is hydrogen-bonded to the 17β-hydroxyl function of levonorgestrel through a water molecule. Several residues contribute to the hydrophobic nature of the ligand-binding cavity and stabilize the position of the levonorgestrel by numerous van der Waals contacts (Fig. 3A).

Superimposing the structure of PRLBD-levonorgestrel (PDB ID: 3D90) over that of PRLBD-progesterone (PDB ID: 1A28) (Fig. 3B) and that of PRLBD-norethindrone (PDB ID: 1SQN) (Fig. 3C) reveals major differences in the interactions involved in the PRLBD-steroid complexes. The Asn719 residue (H3 helix) is hydrogen-bonded to levonorgestrel and norethindrone, whereas it forms no contact with progesterone (Fig. 3, B and C). In the structure of the PRLBD-progesterone, the 19-methyl group contacts the Met759 residue (Fig. 3B). Because of the absence of the 19-methyl group in the levonorgestrel and norethindrone molecules, Met759 (H5 helix) is close to levonorgestrel and norethindrone, permitting numerous additional contacts with the steroid skeleton (Fig. 3, B and C). Because of the 13-ethyl group of levonorgestrel, Met909 (H12 helix) adopts a rotamer distinct from that observed with progesterone and norethindrone (Fig. 3, D–F). Consequently, in the presence of levonorgestrel, Met909 establishes several contacts with Gly722 and Leu726 (H3 helix) and with Met759 (H5 helix) (Fig. 3D), whereas it establishes only one contact with the 13-methyl groups of progesterone (Fig. 3E) and norethindrone (Fig. 3F). The Asn719, Met759, and Met909 residues may therefore make a critical contribution to the high potency of levonorgestrel and/or in levonorgestrel-induced PR activation.

Role of the Asn719 and Met759 Residues in the Progestin Activity. We analyzed the effects of the N719A and M759A mutations on PR activity in response to progesterone- and testosterone-derived progestins. Trans-activation assays showed that progesterone and 19-norprogesterone are still able to activate the mutant PR_{N719A}, but their potencies are

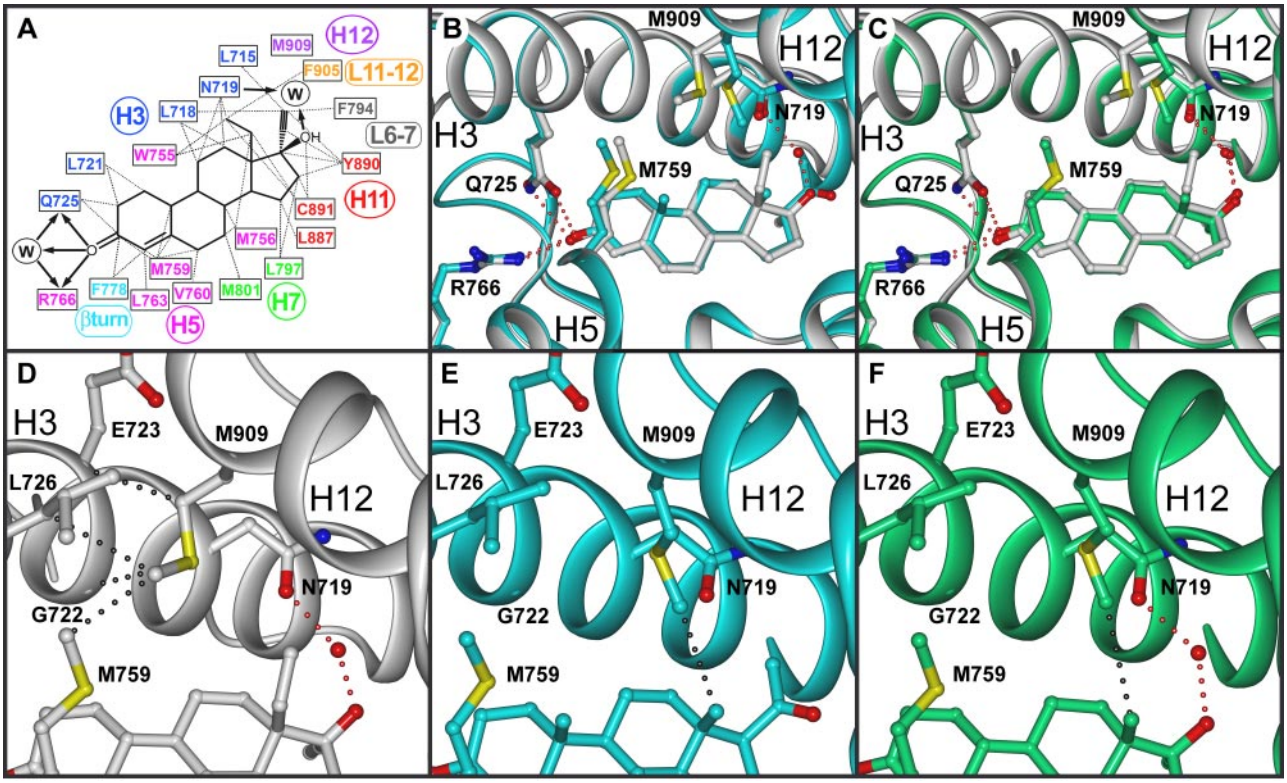

Fig. 3. Crystal structure of the PRLBD in complex with levonorgestrel. A, diagram showing the interactions between PRLBD and levonorgestrel. Hydrogen bonds and van der Waals interactions are depicted as solid black arrows and dotted black lines, respectively. W indicates a water molecule. B, structure of the PRLBD complexed with levonorgestrel (gray) superimposed over that with progesterone (blue). C, structure of the PRLBD complexed with levonorgestrel (gray) superimposed over that with norethindrone (green). Close up of Met909 in the ligand-binding cavity of the PR complexed with levonorgestrel (D), progesterone (E) and norethindrone (F).

lower than those observed with the wild-type receptor (Fig. 4A, Table 1). In contrast, the PR_{N719A} activity induced by 10^{-6} M of 17-hydroxylated progestins (levonorgestrel, ethisterone, and norethindrone) is only 10 to 40% of that induced by 19-norprogesterone (Fig. 4A). Furthermore, at this concentration, the 17-hydroxylated progestins inactivate the 19-norprogesterone-induced activity of PR_{N719A} by 20 to 30% (data not shown). Overall, these findings indicate that the Asn719 residue is critical in the activity of 17-hydroxylated progestins.

Trans-activation assays showed that progestins are still able to activate the PR harboring the M759A mutation. Nevertheless, the PR_{M759A}-activating potencies of the 19-norprogestins (19-norprogesterone, norethindrone, and levonorgestrel) are lower than those observed with the wild-type receptor (Fig. 4B, Table 1). In contrast, the two 19-methyl progestins (progesterone and ethisterone) activate the mutant PR_{M759A} with potencies slightly higher than those observed for the wild-type PR (Fig. 4B, Table 1). It should be noted that levonorgestrel remains the most potent PR_{M759A}-activating progestin. These findings are consistent with the crystal structures, which reveal that the PRLBD makes more stabilizing contacts with the steroid skeleton of compounds with no 19-methyl group.

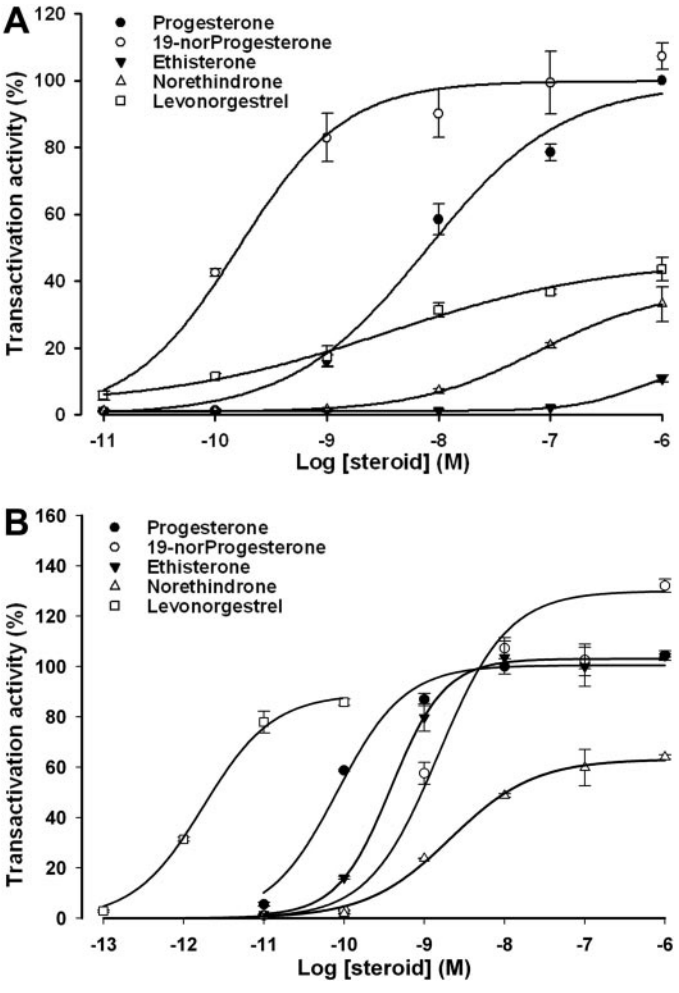

Fig. 4. Trans-activation properties of mutant PRs in response to progestins. HEK 293T cells transiently expressing PR_{N719A} (A) and PR_{M759A} (B) were incubated for 16 h with increasing concentrations of progestins. The trans-activation activities of the mutant PRs were determined from the luciferase activity normalized for β-galactosidase activity. Results are expressed as the percentage of the PR_{N719A} activity induced by 10^{-7} M 19-norprogesterone (A) or of the PR_{M759A} activity induced by 10^{-8} M progesterone (B). Values are mean ± S.E.M. of three separate experiments. A theoretical curve in SigmaPlot has been used to fit the data.

Critical Role of Met909 in the Activity of 13-Methyl and 13-Ethyl Progestin Activity. The structure of the PRLBD complexed with levonorgestrel revealed that Met909 establishes several contacts with Gly722, Leu726, and Met759, whereas it makes only one contact with the 13-methyl group of progesterone and norethindrone (Fig. 3). We therefore wondered whether the capacity of Met909 to establish several stabilizing contacts with other residues of the ligand-binding cavity is responsible for its high potency. Trans-activation assays showed that, at a concentration of 10^{-7} M, the 13-methyl and 13-ethyl derivatives (progesterone, 19-norprogesterone, ethisterone, norethindrone, and levonorgestrel) are unable to activate PR_{M909A} (Fig. 5A). In contrast, the synthetic compound 18-oxo-18-vinylprogesterone, characterized by having a 13-enone group (Souque et al., 1995), activates both PR and PR_{M909A} (Fig. 5A).

Thus, we wondered whether 13-methyl and 13-ethyl progestins are able to bind the PR_{M909A}. The binding studies presented in Fig. 5B show that 10^{-9} M [^{3}H]progesterone binds to both PR and PR_{M909A}. Furthermore, progesterone and levonorgestrel (10^{-6} M) were both able to inhibit [^{3}H]progesterone binding to PR and to PR_{M909A} (Fig. 5B). Thus, PR_{M909A} retains the ability of PR to bind progesterone and levonorgestrel.

Limited proteolysis assays with the PR have revealed a major 30-kDa fragment that encompasses the LBD (Allan et al., 1992). They have also shown that the resistance of this 30-kDa fragment was increased in response to ligand binding, indicating a ligand-induced conformational change of the LBD. We therefore performed limited proteolysis assays to find out whether the mutation modifies the LBD conformation and whether progestins were able to increase resistance of the PR_{M909A} to trypsin, as they do for the wild-type receptor. Incubating ^{35}S-labeled PR and PR_{M909A} with 5 μg/ml trypsin generates a major 30-kDa fragment, and two minor fragments of 33 and 26 kDa (Fig. 5C). These fragments were completely digested by 25 μg/ml trypsin, suggesting that the M909A mutation does not modify the sensitivity of the PRLBD to trypsin and so does not modify the LBD conformation. It is noteworthy that levonorgestrel (Fig. 5C) and progesterone (data not shown) increased the resistance to trypsin of the 26- and 30-kDa fragments produced from both PR and PR_{M909A}. These results confirm that levonorgestrel and progesterone bind to $PR_{M909A,}$ and modify the LBD conformation, rendering it more resistant to proteolysis.

Because the 13-methyl and 13-ethyl progestins bind to PR_{M909A} but are unable to activate it at the 10^{-7} M concentration, we further investigated whether progesterone and levonorgestrel inhibit the 18OVP-induced PR_{M909A} activity. At the 10^{-5} M concentration, progesterone inhibits the 18OVP-induced PR_{M909A} activity by 60%. At the 10^{-6} M concentration, levonorgestrel inhibits the 18OVP-induced PR_{M909A} activity by only 30%. On the other hand, at the 10^{-5} M concentration, levonorgestrel does not inhibit the 18OVP-induced PR_{M909A} activity, whereas it does activate this mutant (Fig. 5D). These results indicate that progesterone displays antagonist activity, whereas levonorgestrel behaves as a partial agonist when bound to PR_{M909A}.

Finally, we analyzed the ability of PR and PR_{M909A} to recruit the transcriptional coactivator TIF1α. GST pull-down assays show that incubation of PR with 10^{-6} M levonorgestrel or 18OVP promoted its interaction with TIF1α (Fig. 5E), confirming the agonist properties of these two molecules on the PR. In contrast, the recruitment of TIF1α by PR_{M909A} occurs in the presence of 10^{-6} M 18OVP, but not with 10^{-6} M levonorgestrel. These results are consistent with the observation that 18OVP is able to activate the mutant PR_{M909A} and that levonorgestrel behaves as a partial agonist when bound to PR_{M909A}.

Discussion

We report here the crystal structure of the PRLBD associated with levonorgestrel, a very potent 18,19-dinorprogestin derived from testosterone, that is characterized by a 13-ethyl group. By means of mutagenesis analysis and functional studies, we show that the Met909 residue in the H12 helix plays a crucial role in the PR activation. We point out that the contacts involved in the PR activation depend on the presence of a 13-ethyl (levonorgestrel) or 13-methyl group (progesterone) on the steroid skeleton. We identified Met909

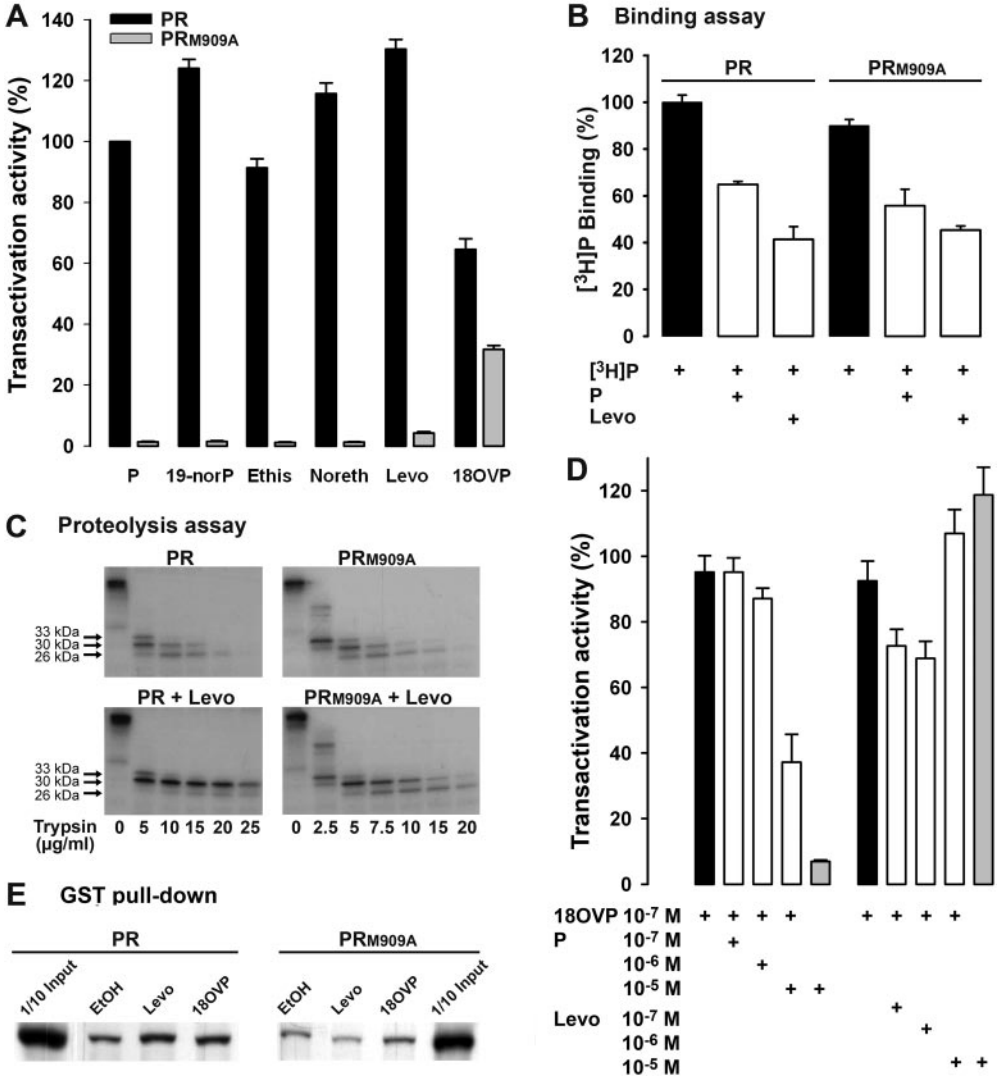

Fig. 5. Characteristics of the mutant PR_{M909A}. A, trans-activation activity of PR_{M909A} in response to progestins. HEK 293T cells transiently expressing PR or PR_{M909A} were incubated for 16 h with 10^{-7} M progestins (P, progesterone; 19-norP, 19-norprogesterone; Ethis, ethisterone; Noreth, norethindrone; Levo, levonorgestrel; 18OVP, 18-oxo-18-vinylprogesterone). The PR trans-activation activities were determined from the luciferase activity normalized for β-galactosidase activity. Results are expressed as a percentage of PR activity in response to 10^{-7} M progesterone. Values are mean $\pm$ S.E.M. of three separate experiments. B, steroid binding experiments. HEK 293T cells transiently expressing PR or PR_{M909A} were incubated for 30 min at 20°C with 10^{-9} M [^{3}H]progesterone with or without 10^{-6} M progesterone or levonorgestrel. Cells were rinsed rapidly, and the radioactivity was counted after ethanol extraction. Results are expressed as the percentage of [^{3}H]progesterone binding to PR. C, limited proteolysis assays. [^{35}S]-labeled PR and PR_{M909A} were produced by translation in vitro, and incubated for 10 min at 20°C without or with 10^{-6} M levonorgestrel, and then for 10 min at 20°C without or with increasing concentrations (2.5–25 μg/ml) of trypsin. The digestion products were analyzed by SDS-page and autoradiographed. The molecular mass of the digested fragments are indicated on the left-hand side of the figure. D, ability of progesterone and levonorgestrel to inhibit the 18OVP-induced PR_{M909A} activity. HEK 293T cells transiently expressing PR_{M909A} were incubated for 16 h with 10^{-7} M 18OVP, progesterone or levonorgestrel alone or with 10^{-7} M 18OVP in the presence of increasing concentrations of progesterone or levonorgestrel. The PR trans-activation activities were determined from the luciferase activity normalized for β-galactosidase activity. Results are expressed as a percentage of PR_{M909A} activity induced by 10^{-7} M 18OVP alone. Values are mean $\pm$ S.E.M. of three separate experiments. E, interaction of TIF1α with PR or PR_{M909A}. GST fusion proteins, which had previously been coupled to Sepharose glutathione beads, were incubated with [^{35}S]methionine-labeled PR and PR_{M909A} in the absence (EtOH) or presence of 1 μM levonorgestrel or 18OVP for 20 min at 20°C. The glutathione Sepharose beads were washed and boiled in Laemmli buffer. The samples were then analyzed by SDS-polyacrylamide gel electrophoresis, followed by autoradiography.

together with Asn719 and Met759 as modulators of progestin potency and established a clear structure-activity relationship among progestins.

The crystal structures of the human PRLBD associated with various progestins have been solved (Williams and Sigler, 1998; Madauss et al., 2004; Zhang et al., 2005). Neither the contact(s) between progestins and the ligand-binding cavity required to trigger PR activation, nor the structural elements that modulate progestin activity, were identified. The structure of the PRLBD-levonorgestrel complex, reported in this study, reveals a contact between the Asn719 of the H3 helix and the 17β-hydroxyl group of levonorgestrel. A similar contact has been identified in the structure of PRLBD complexed with norethindrone, a progestin that also has a 17β-hydroxyl function (Madauss et al., 2004), but not with progesterone, which is characterized by a 17-methyl ketone function (Williams and Sigler, 1998). Accordingly, the N719A mutation does not affect the full agonist character of progesterone, whereas it reduces the activity of the 17β-hydroxylated progestins and makes them partial agonists. Thus, the PR activation induced by progesterone is not Asn719 dependent. Conversely, Asn719 contributes to the PR activation by the 17β-hydroxylated progestins, but its role is not essential. It is noteworthy that Asn719 is conserved in the other 3-oxosteroids receptors. The strong hydrogen bond between the Asn705 of the AR, and the 17β-hydroxyl group of androgen ligands plays a crucial role in androgen binding and AR activation (Matias et al., 2000; Poujol et al., 2000; Pereira de Jésus-Tran et al., 2006). In a similar way, GR and MR activation are dependent on the contact between the 21-hydroxyl group of ligands, and the GRN564 and MRN770 (Fagart et al., 1998; Hellal-Levy et al., 1999; Lind et al., 2000; Bledsoe et al., 2002; Bledsoe et al., 2005). Thus, unlike the AR, GR, and MR, contact between progestins and the asparagine of the H3 helix is not a prerequisite for PR activation.

In the structures of the PRLBD complexed with levonorgestrel, the Met909 residue of the H12 helix adopts a rotamer distinct from that observed with progesterone and norethindrone. A contact between the Met909 and the 13-methyl group is observed in the complexes involving progesterone and norethindrone (Williams and Sigler, 1998; Madauss et al., 2004). Because of its 13-ethyl substituent, levonorgestrel is unable to contact the Met909. In contrast, in this complex, the Met909 makes numerous stabilizing contacts with residues of the ligand-binding cavity (Gly722, Leu726, and Met759). We show that, at the concentration of 10^{-7} M, progestins carrying either a 13-methyl or a 13-ethyl group are unable to activate PR_{M909A}. Nevertheless, they do bind to this mutant and enhance LBD resistance to proteolysis as they do for the wild-type receptor. They also display antagonist (progesterone) or partial agonist (levonorgestrel) properties because they inhibit the 18-OVP induced PR_{M909A} activity. Accordingly, PR_{M909A} complexed with levonorgestrel is unable to recruit the transcriptional coactivator TIF1α. We therefore propose that Met909 plays a crucial role in activating PR by both 13-ethyl and 13-methyl progestins.

Levonorgestrel is more potent than norethindrone. It is remarkable that these two molecules differ only by their C13 substituents, an ethyl and a methyl group for levonorgestrel and norethindrone, respectively. The network of contacts involving Met909 and residues of the helices 3 and 5 is probably responsible for the high potency of levonorgestrel. Nestorone, a molecule characterized by a 13-methyl and a 17-methyl ketone function, is a potent progestin. It is noteworthy that the replacement of its 13-methyl by a 13-ethyl increases its affinity for the PR and confers to this molecule a 3- to 10-fold higher potency (Tuba et al., 2000). It is likely that 13-ethyl nestorone derivative can create a network of stabilizing contacts as does levonorgestrel. Thus, we propose that Met909 is responsible for the high potency of 13-ethyl progestins regardless the nature of their C17-substituents.

We also show that 19-norprogestins are more potent than molecules with a 19-methyl group. The structure of the PRLBD complexed with progesterone reveals that the 19-methyl group contacts the Met759 residue of the H5 helix (Williams and Sigler, 1998). In the complexes with levonorgestrel and norethindrone (Madauss et al., 2004), Met759 is close to the ligand, permitting numerous stabilizing contacts with the steroid skeleton. Accordingly, the M759A mutation markedly reduces the potency of 19-norprogestins but not that of progestins with a 19-methyl group. Thus, the close contact between Met759 and the steroid skeleton is responsible for the high potency of 19-norprogestins.

From this study, it can be concluded that the Met909 of the helix 12 plays a key role in PR activation by both testosterone- and progesterone-derived progestins. We also show that the higher potency of 13-ethyl progestins compared with 13-methyl progestins is due to the Met909, whereas the high potency of 19-norprogestins is related to Met759. Our findings provide a structural guideline for the rational synthesis of potent PR agonist and antagonist ligands that could have therapeutic uses in women's health.

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