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Development of Pure Prolactin Receptor Antagonists*

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Prolactin (PRL) promotes tumor growth in various experimental models and leads to prostate hyperplasia and mammary neoplasia in PRL transgenic mice. Increasing experimental evidence argues for the involvement of autocrine PRL in this process. PRL receptor antagonists have been developed to counteract these undesired proliferative actions of PRL. However, all forms of PRL receptor antagonists obtained to date exhibit partial agonism, preventing their therapeutic use as full antagonists. In the present study, we describe the development of new human PRL antagonists devoid of agonistic properties and therefore able to act as pure antagonists. This was demonstrated using several *in vitro* bioassays, including highly sensitive assays able to detect extremely low levels of receptor activation. These new compounds also act as pure antagonists *in vivo*, as assessed by analyzing their ability to competitively inhibit PRL-triggered signaling cascades in various target tissues (liver, mammary gland, and prostate). Finally, by using transgenic mice expressing PRL specifically in the prostate, which exhibit constitutively activated signaling cascades paralleling hyperplasia, we show that these new PRL analogs are able to completely revert PRL-activated events. These second generation human PRL antagonists are good candidates to be used as inhibitors of growth-promoting actions of PRL.

The development of prolactin (PRL)[1] antagonists has been an emerging field of research since the mid 1990s. These investigations have been performed to better understand the increasing body of evidence that PRL is able to promote tumor growth of some of its target tissues, as has been recognized for a long time with respect to mammary tumors in rodents (for reviews see Refs. 1 and 2). As an illustration of this, it was recently shown that the appearance of genetically induced mammary tumors in mice is delayed in PRL-deficient mice (3), whereas PRL transgenic mice spontaneously develop mammary neoplasia (4). In contrast, the involvement of PRL in human breast tumors has always been controversial, primarily because no strong correlation between the circulating levels of PRL and the risk to develop breast cancer could ever be established, except for more recent studies (5–7). In addition, the lack of any clinical improvement of breast cancer patients treated with dopamine agonists (which lower circulating levels of PRL) rapidly reduced the interest of oncologists with respect to a potential role of PRL in the development of breast cancer (8–10). A number of recent observations argues strongly that the role of PRL in the progression of breast cancers (and maybe of other cancers) needs to be reconsidered. First, at the epidemiological level, the largest study ever performed (Nurse Health study) clearly shows a positive correlation between high normal PRL levels and the risk of breast cancer in post-menopausal women (11). Second, and even more important is the discovery that PRL is expressed by many extra-pituitary sites, including mammary epithelial cells, and that this locally produced hormone stimulates cell proliferation via an autocrine-paracrine loop in many species, including humans (see Refs. 12–14 and for reviews see Refs. 15 and 16). Third, the PRL receptor (PRLR) is expressed in virtually all mammary epithelial cells, and its level of expression, at least at the RNA level, is in general higher in tumors compared with the adjacent normal tissue, arguing for an increased sensitivity of tumor cells to the hormone (17–19). Fourth, recent observations show that PRL activates the expression of various proteins known to be key players in breast cancer progression, such as cyclin D1 (20, 21), or insulin-like growth factor-2, recently identified as a putative relay of some PRL actions in the breast (22, 23).

The only anti-PRL drug currently available for clinical use is a family of dopamine agonists, the prototype of which is bromocriptine. Like dopamine, these compounds efficiently inhibit PRL synthesis and release from its major source of production, the pituitary. However, because extrapituitary PRL production is regulated by mechanisms and molecules still unknown, but clearly different compared with those acting in the pituitary (24), dopamine agonists are unable to block PRL secretion from extrapituitary tissues. The increasing evidence that locally produced, maybe even more than pituitary-produced, PRL might play a key role in promoting tumor growth encouraged the search for alternative strategies to counteract this proliferative effect. One such approach is the development of specific PRLR antagonists.

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[1] The abbreviations used are: PRL, prolactin; GH, growth hormone; PRLR, prolactin receptor; h, human; r, rat; WT, wild type; STAT, signal transducer and activator of transcription; MAPK, mitogen activated protein kinase; ELISA, enzyme-linked immunosorbent assay; HRP, horseradish peroxidase; LHRE, lactogenic hormone-response element.

The closely related growth hormone (GH) receptor along with the PRLR were recognized as the initial members of the hematopoietic cytokine receptor superfamily, the cDNAs of which were cloned 15 years ago (25, 26). The mechanism of ligand-induced activation of these receptors has been widely studied by us (27, 28) and others (29). The active form of PRL and GH receptors is a homodimer composed of two identical membrane chains, each of which interacts with opposing sides of the hormone, referred to as binding sites 1 and 2. Because these ligands bind first one receptor chain via their binding site 1, to form an inactive intermediate 1:1 complex, and then to a second receptor chain, to form an active 1:2 complex, the most classical strategy to develop PRL or GH antagonists has been based on the rational design of ligands with impaired binding site 2. The prototype of such mutations is the substitution of the conserved helix 3 glycine for larger side chain residues, such as arginine or tryptophan, which sterically hinder the binding site 2 (30, 31). So-called G120R-hGH or G129R-hPRL analogs were found to be potent antagonists of their respective receptors in many *in vitro* bioassays (30, 32), including proliferation and PRL receptor-mediated activation of signaling cascades in human breast cancer cell lines *in vitro* (33–35). In human mammary tumor cell lines, the PRLR antagonist G129R-hPRL was also reported to induce apoptosis (35, 36), to inhibit PRL activation of transcription factor STAT3 (37), and to reduce tumor growth *in vivo* (38). Despite these encouraging reports arguing for the potential interest of G129R-hPRL as a potent inhibitor of PRL actions in the context of breast cancer, our recent observations clearly show that this PRL analog has its disadvantages, because it fails to antagonize PRL in many situations. Reminiscent to the problem encountered by many selective estrogen receptor modulators, G129R-hPRL exhibits some residual agonistic activity, which in some instances predominates over its antagonistic properties. We have recently shown, by comparing several *in vitro* bioassays, that the more sensitive the bioassay, the more pronounced is this residual agonism (39). Because the mechanism of action of such PRLR antagonists is to compete with WT PRL for binding to the receptor, this implies that the antagonists must be used in molar excess, *i.e.* at concentrations at which the residual agonistic activities tend to predominate. This is clearly the case in transgenic mice, which express G129R-hPRL at concentrations 10–100-fold higher than endogenous PRL.[2] These mice fail to exhibit any of the phenotypes observed in PRLR knockout mice (40), such as female sterility and mammary gland failure, but instead exhibit certain phenotypes reminiscent of moderate hyperprolactinemia, such as constitutive MAPK activation in the prostate (see below).

These and other observations clearly demonstrate that G129R-hPRL is not a final clinically usable product but requires further improvements. In the present study, we describe the development of pure PRLR antagonists, *i.e.* second generation compounds completely devoid of any residual agonistic activity in all *in vitro* bioassays used, including the most sensitive cell proliferation assays. We also provide evidence that these new PRL analogs are potent inhibitors of PRL actions *in vivo*, including the effects induced by locally produced hormone in transgenic mice expressing PRL only in the prostate.

EXPERIMENTAL PROCEDURES

Materials

Reagents—Culture media, fetal calf serum, geneticin (G-418), trypsin, and glutamine were purchased from Invitrogen. Luciferin and cell lysis buffer were from Promega (Madison, WI), and luciferase activity was measured in relative light units (Lumat LB 9501, Berthold, Nashua, NH). IODO-GEN was purchased from Sigma, and carrier-free Na[^{125}I] was obtained from Amersham Biosciences. Salts were high grade purified chemicals purchased from Sigma or Merck. Oligonucleotides were from Eurogentec (Liège, Belgium). Bromocriptine was purchased from Sigma (catalog number B2134).

Hormones—In this study, we used exclusively recombinant proteins as follows: WT hPRL (41), the molecular mimic of phosphorylated PRL named S179D-hPRL (42); the first generation antagonist G129R (Gly^{129} replaced with Arg) (31); and the two new antagonists constructed in this study, Δ1–9-G129R-hPRL and Δ1–14-G129R-hPRL, characterized by the deletion of the 9 or 14 N-terminal residues, respectively. The pT7L expression vector used for expression of all hPRL analogs was described previously (41). Recombinant WT and mutated hPRL were produced in *Escherichia coli* as inclusion bodies and purified as shown in our former publications (31, 32, 41, 43). Briefly, protein purification was performed using ion exchange columns (Hi Trap Q-Sepharose) purchased from Amersham Biosciences. The hGH antagonist G120K-hGH (Gly^{120} replaced with Arg) was kindly provided by Sensus Drug Development Corp. (Austin, TX).

Antibodies, RIA, ELISA—Antibodies used in this study are as follows: polyclonal anti-hPRL (A569, Dako), a monoclonal anti-active MAPK (directed against Thr^{202}/Tyr^{204}-phosphorylated MAP kinases 1 and 2, also referred to as anti-active Erk1/2; Cell Signaling, catalog number 9106), polyclonal anti-MAPK1/2 (Upstate Biotechnology, Inc., catalog number 06-182), polyclonal anti-phosphorylated STAT5A/B (Upstate Biotechnology, Inc., catalog number 06-867), polyclonal anti-STAT5 (C-17; Santa Cruz Biotechnology), monoclonal anti-phosphorylated STAT3 (Upstate Biotechnology, Inc., clone 9E12), and polyclonal anti-STAT3 (C-20; Santa Cruz Biotechnology).

Quantification of circulating hPRLs after hormone injections in mice was performed in serum using a radioimmunoassay (IRMA, Immunotech, France) or a human PRL-specific enzyme-linked immunosorbent assay (Prolactin Elisa kit, Diagnostic Biochem Dbc. Canada Inc., Ontario, Canada) that we modified as described below. The absence of cross-reaction with endogenous murine PRL or transgenic rPRL was assessed using control sera of non-treated mice or purified hormones.

Animals—Mice used in this study were WT Balb/c-J mice (Charles River Laboratories, l'Arbresle, France) or mice transgenic for rat PRL under the control of the probasin gene promoter, which directs specific expression of the transgene in the prostate (44). Non-transgenic littermates were used as controls when appropriate. Mice were housed and experimental protocols were in agreement with the procedures established by the local ethical committee.

Software—All curves presented were analyzed and performed with GraphPad Prism version 3.02 for Windows (GraphPad Software, San Diego, www.graphpad.com). Autoradiographies (Western blot) were analyzed using Scion Image software (Scion Corp.).

Methods

Site-directed Mutagenesis—Construction of expression plasmids encoding Δ1–9-hPRL and Δ1–14-hPRL analogs was performed using PCR; plasmid pT7L-hPRL (41) was used as template. Sequences of 5′ oligonucleotides correspond to the 5′ sequence of the hPRL cDNA lacking the 9 (Δ1–9-hPRL) or 14 (Δ1–14-hPRL) N-terminal codons. A unique *Nde*I restriction site (CATATG) containing the ATG codon (methionine initiator) was inserted in the 5′ oligonucleotide, as follows: Δ1–9, GGCAT-ATGCGATCCCAGGTGACCCTTCG; Δ1–14, GGCATATGCTTCGAGA-CCTGTTTGACC. The 3′ oligonucleotide was identical for both analogs; it corresponds to a sequence in the non-coding region of the hPRL cDNA, located 3′ of the unique *Hin*dIII restriction site: 5′-CTGTTA-CACCCACGCATGG-3′. The PCR was performed as follows: 200 μM dNTP, 45 μM $MgCl_2$, 1.5 μl of *Taq* polymerase (5 units/μl), PCR buffer, 10 ng of template (plasmid pT7L-hPRL), 20 pmol of each primers. PCR was performed for 25 cycles: 94 °C (30 s), 56 °C (30 s), and 72 °C (1 min). PCR products were subcloned into TA cloning vector (pCR II.1, Invitrogen), and then recombinant TA plasmids were digested using *Nde*I/*Hin*dIII restriction enzymes, and purified inserts were ligated into pT7L plasmid linearized at identical sites. After transformation, *E. coli* BL21(DE3) colonies were analyzed for their DNA content; plasmids were extracted and digested to confirm the presence of expected inserts and then sequenced to check the expected mutations.

Expression plasmids encoding analogs Δ1–9-G129R-hPRL and Δ1–14-G129R-hPRL were constructed by substituting the *Eco*RI-*Bgl*II fragment from pT7L-G129R-hPRL plasmid (containing the G129R mutation) (31) for the corresponding *Eco*RI-*Bgl*II fragment in pT7L-Δ1–9-hPRL and pT7L-Δ1–14-hPRL expression vectors. Clones obtained

[2] S. Bernichtein, C. Kayser, J. J. Kopchick, P. A. Kelly, and V. Goffin, unpublished data.

were analyzed for the presence of the insert and then sequenced to check the expected mutations. Analog expression was performed using BL21(DE3) bacteria as described above.

Biochemical Characterization of hPRL Analogs—The content of α-helical structure was calculated from circular dichroism spectra obtained as described previously (31, 43, 45, 46). The apparent molecular mass was estimated from the elution volume on gel filtration chromatography (Sephacryl S-100 or S-200 loaded into a C16/70 column; Amersham Biosciences) with respect to the elution volume of standard protein as described elsewhere (46).

Binding Assays

Binding affinities of hPRL analogs were determined using cell homogenates of HL5 cells (293 HEK cells stably expressing the human PRLR), following a procedure described previously (46). Briefly, hPRL was iodinated using IODO-GEN, and its specific activity was in the range of 40–50 μCi/μg. Binding assays were performed overnight at room temperature using 150–300 μg of cell homogenate protein in the presence of 30,000 cpm ^{125}I-hPRL and increasing concentrations of unlabeled competitor (WT or mutated hPRL). Results presented are representative of at least three independent experiments performed in duplicate. The relative binding affinity of analogs was calculated as the ratio of their IC_{50} with respect to that of WT hPRL.

Cell-based Bioassays

Established Bioassays—PRL analogs were analyzed using four cell lines, following experimental procedures (media, stimulation time, etc.) detailed in our recent publication describing the two new homologous bioassays developed for human lactogens (39). Proliferation studies were performed using the reference assay for lactogens, Nb2 cells (47, 48), or the proliferation assay that we recently established using Ba/F-03 cells stably transfected with the expression plasmid encoding the human PRLR (referred to as Ba/F-LP cells). The transcriptional study was based on the activation of the lactogenic hormone-response element (LHRE)-luciferase reporter gene, performed using the HL5 clone as recently described (39, 42, 46). Finally, we used T47D human breast cancer cell lines to assess signaling events triggered by the PRLR, using procedures described under "Signaling Studies" below.

Mouse PRLR-mediated Transcriptional Bioassay—Prior to the analysis of the new antagonists *in vivo*, their ability to antagonize PRL-induced effects involving the mouse PRLR was tested *in vitro*. Similarly to the assay developed for the human PRLR (39), 293 HEK cells were stably transfected using plasmids encoding the long isoform of mouse PRLR (49) and the LHRE-luciferase reporter vector. One stable clone isolated by geneticin selection was selected for functional studies and referred to as ML-F5 (Mouse PRLR-Luciferase, clone F5). Experimental procedures are identical to those described previously (39) for the same assay involving the human receptor.

For all bioassays, agonistic properties of the various ligands were assessed by testing dose response of these hormones alone, and their potential antagonistic activity was tested by competing a fixed concentration of WT hPRL with increasing amounts of the putative antagonists, as indicated in the legends to figures.

Signaling Studies—Signaling studies were performed using lysates of T47D cells or of mouse tissues harvested as described below. T47D cells were starved overnight in fetal calf serum-free medium before hormonal stimulation. Stimulations were performed as indicated in the figures. Cells were then washed twice with ice-cold PBS (34), scraped, and centrifuged, and the pellet was kept frozen until used. Cells were solubilized in 1 ml of lysis buffer (30 min rotation at 4 °C; Ref. 34), and lysates were centrifuged for 10 min at 13,000 $\times$ *g*, and then the protein content of supernatants was measured by the Bradford assay.

For STATs immunoprecipitation studies, 1–2 mg of protein lysates were incubated with polyclonal anti-STAT5 or anti-STAT3 (5 μl/ml). After overnight rotation at 4 °C, immune complexes were captured using 20 μl of protein A-Sepharose slurry (Amersham Biosciences) for 1 additional hour at 4 °C. Protein A complexes were precipitated by centrifugation, and pellets were washed 3 times in lysis buffer and boiled in 15 μl of reducing SDS sample buffer for 5 min at 95 °C. Finally, immunoprecipitated samples were analyzed using 7.5% SDS-PAGE. Analysis of MAPK activation was performed on total lysates of T47D cells using 50–100 μg of protein per lane on 10% SDS-PAGE. Electrophoretic transfer onto nitrocellulose membranes (Bio-Rad) and membrane treatments were performed as described earlier (34). Immunoblotting was performed using antibodies directed against phosphorylated STAT5 or STAT3 (1:500 dilution), active Erk1/2 (1:1,000 dilution), total MAPK (1:1,000 dilution), and total STAT3 or STAT5 (1:1,000 dilution). Procedures for membrane washing, incubation with HRP-coupled secondary antibodies, enhanced chemiluminescence, autoradiography, stripping, and re-hybridization were as described (34). Densitometric analysis of autoradiographies was performed using the image analysis software Scion Image (Scion Corp.).

The activation of STAT5 was also analyzed using the "TransAM STAT" kit developed by Eppendorf Array Technologies (Namur, Belgium) and purchased from ActiveMotif (San Diego). We strictly followed the instructions provided by the manufacturer. The principle of this assay is that STAT-specific DNA oligonucleotides were immobilized onto the bottom of 96-well plates, and then 5–10 μg of cell or tissue lysates were incubated in each well (in triplicate), and after appropriate treatments including washings, active STATs (STAT5 in our case) were detected using HRP-conjugated antibodies specifically interacting with activated forms of each STAT.

Animal Studies

For prostate studies, we used probasin-rPRL transgenic mice, in which overexpression of rat PRL was restricted to the prostate by using the probasin minimal promoter to drive the expression of the rat PRL gene. Probasin is an androgen-dependent prostate protein. Transgenic probasin rPRL males have been shown to develop dramatic prostate hyperplasia (44). Transgenic males (6–9 months of age) were injected (subcutaneously) with various amounts of hPRL, alone or in combination with hPRL analogs as indicated in the figure legends. One hour after the injection, mice were sacrificed by cervical dislocation and dissected rapidly to harvest liver or prostate tissues. Dissection of the urinary tract to isolate dorsolateral and ventral lobes was performed following the procedure extensively described by Kindblom *et al.* (44). For liver studies, Balb/c-J WT females (8 weeks) were used and treated the same way. For mammary gland studies, lactating WT mice (13–15 days of lactation) were first injected with 200 μg of bromocriptine dissolved in ethanol and then diluted in NaCl 0.9% (Sigma) to markedly decrease pituitary PRL production. Five hours later, they were injected with hPRL, alone or in combination with the antagonists. Mice were sacrificed after 30 min of treatment, and the 4th mammary glands were rapidly harvested.

Freshly harvested prostate lobes, livers and mammary glands were immediately placed in ice-cold lysis buffer, dissected, and cut into small pieces rapidly, and then lysed using a Polytron (3 times for 5–10 s). Tissue lysates were centrifuged, and supernatants were snap-frozen and kept at −80 °C until used for signaling experiments.

Quantification of Antagonists in Serum (ELISA)

Quantification of hPRL analogs was performed using the human PRL-specific enzyme-linked immunosorbent assay purchased from Diagnostic Biochem Dbc (Prolactin Elisa kit), with the exception that polyclonal anti-hPRL antibody (A569, Dako; 5 μl/well of 1:500 diluted antibody) was substituted for the secondary HRP-conjugated monoclonal anti-hPRL antibody provided in the kit. Detection was then performed by adding HRP-conjugated anti-rabbit antibody (1:5,000 dilution).

RESULTS

Production and Characterization of hPRL Analogs in E. coli

All hPRL mutants used in this study were produced in bacteria as inclusion bodies as reported previously (31, 32, 43, 46). N-terminal deleted G129R-hPRL mutants refolded correctly as assessed by their helical content around 50% and their apparent molecular mass similar to hPRL (not shown), suggesting that shortening the N terminus does not disturb global protein conformation. As reported (68) for N-terminally deleted hPRL, the only repeated difference between mutated and WT hPRL was that N-terminal deletions tended to increase the monomeric/multimeric ratio observed after protein refolding. We believe that removal of the two N-terminal cysteines (Cys^4–Cys^{11}) prevents formation of covalent multimers involving intermolecular disulfide bonding between these residues.[3] Production of S179D was less efficient, probably because of misfolding as reported previously (42).

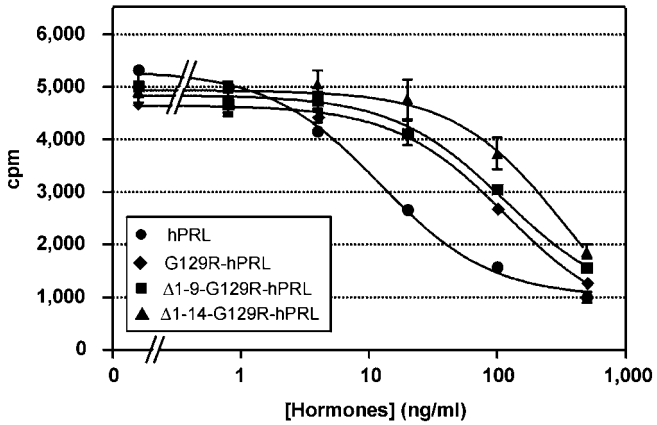

FIG. 1. **Binding assay of G129R-containing hPRL analogs.** Representative competition curves performed in triplicate for WT and the three hPRL analogs containing the G129R mutation are shown. The three curves of G129R-containing mutants are displaced to the right by ~1 order of magnitude compared with WT hPRL, reflecting ~10-fold lower affinity for the human receptor.

Binding Studies

The affinity of WT hPRL for the human PRLR as calculated by Scatchard analysis indicated a K_d of 3.4 (±1.3) × 10^{-10} M (46). The affinity of the G129R-containing hPRL analogs for the human PRLR was estimated by their ability to compete ^{125}I-hPRL for binding to this receptor and quantified by the IC_{50} of displacement curves. As shown in Fig. 1, the three curves are displaced to the right by ~1 order of magnitude compared with WT hPRL, reflecting ~10-fold lower affinity for the receptor as reported previously (46) for G129R-hPRL. Averaged from three independent experiments, IC_{50} values were 166 ± 47 ng/ml for Δ1–9-G129R-hPRL and 187 ± 49 ng/ml for Δ1–14-G129R-hPRL, compared with 18 ± 5 ng/ml for WT hPRL.

This experiment shows that the three analogs harboring the G129R substitution have a similar affinity for the human PRLR.

Cell-based Bioassays

All bioassays were performed using Δ1–9-G129R-hPRL, Δ1–14-G129R-hPRL, and G129R-hPRL in the same experiment. In some experiments, the hGH antagonist G120K-hGH and/or the S179D-hPRL analog were also included as controls (not shown).

Antagonism—The antagonistic properties of hPRL analogs were performed using three types of assay: transcriptional activation monitored by luciferase induction in the HL5 clone, cell proliferation using the Ba/F-LP assay, and PRLR-signaling cascades using the human breast carcinoma cells T47D.

In agreement with their relative affinity for the hPRLR, the antagonistic properties of the three analogs were very similar in the luciferase assay, although Δ1–9-G129R-hPRL and Δ1–14-G129R-hPRL repeatedly were slightly less efficiency than G129R-hPRL (Fig. 2*A*). We have shown previously that the hGH analog G120K-hGH is also a potent antagonist in this assay (50), whereas the analog S179D-hPRL is not an antagonist but is instead a super-agonist (42). Similar observations were confirmed in this study (not shown). By using Ba/F-LP cells, all three G129R-containing analogs displayed similar antagonistic activities, meaning that efficient competition with WT hPRL required high molar excess of the analog being used (10–50-fold), irrespective of N-terminal deletions (Fig. 2*B*). With respect to the double mutants, competitive inhibition of WT hPRL-induced activity presumably reflects a true phenomenon of antagonism, because these analogs are devoid of intrinsic agonistic effect (see below). In contrast, because G129R-hPRL displays significant agonistic activity in this assay (39), the inhibitory effect observed in competition assays presumably reflects a combination of real antagonism and self-antagonism (28, 31, 42). Finally, the antagonistic activity of the new analogs was further confirmed by their ability to inhibit hPRL-induced activation of the MAP kinases Erk1/2 (not shown) and Stat5 (Fig. 2*C*) in T47D breast cancer cells.

In summary, these experiments show that the two new analogs Δ1–9-G129R-hPRL and Δ1–14-G129R-hPRL exhibit antagonistic properties very similar to those of G129R-hPRL analog, and thus do not provide any improvement with respect to this particular parameter.

Agonism—As reported earlier (32, 46), the agonistic activity of G129R-hPRL is extremely reduced in the luciferase assay, with a maximal level <2% of hPRL activity. None of the new antagonists induced any detectable level of luciferase activity, even when tested at extremely high concentrations (up to 50 μg/ml) (Fig. 3*B*). Similarly, we were unable to detect any signal induced by any of the G129R-containing mutants when monitoring PRLR-triggered signaling cascades in T47D cells, *e.g.* MAPK (data not shown) or STAT5 activation (Fig. 3*A*). We recently showed (39) that the agonistic properties of hPRL analogs depends on assay sensitivity, *i.e.* such activity is not detectable in the less sensitivity assays (such as the luciferase or signaling assays) but is clearly displayed in the highly sensitive assays. Therefore, it was of primary interest to assess the residual agonism of the new analogs using the two most sensitive bioassays currently available, involving Ba/F-LP or Nb2 cells. With respect to the former, the curve obtained for G129R-hPRL is displaced to the right by ~2 log units and achieved sub-maximal (50–80%) levels compared with hPRL as reported before (39). At very high concentrations, hPRL and G129R-hPRL displayed bell-shaped curves, as typically observed when using these ligands (28). Both Δ1–9-G129R-hPRL and Δ1–14-G129R-hPRL failed to show any agonistic activity, even at concentrations as high as 10 μg/ml (Fig. 3*C*). In the Nb2 assay, the agonistic dose-response curve obtained with G129R-hPRL is shifted by 2 log units to the right compared with WT hPRL, with maximal effect achieved at ~1 μg/ml (31, 42). Once again, this agonistic activity is totally abolished when the N-terminal region of G129R-hPRL is deleted (meaning in Δ1–9-G129R-hPRL and Δ1–14-G129R-hPRL analogs), and this was true even at concentrations up to 4 orders of magnitude higher than the concentration leading to maximal activity of WT hPRL (1 ng/ml *versus* 10 μg/ml) (Fig. 3*D*).

These experiments show that deletion of the N-terminal tail combined with the G129R mutation generates pure antagonists, completely devoid of the agonistic effect observed with G129R-hPRL, even in the most sensitive bioassays.

Animal Studies

Because both Δ1–9-G129R-hPRL and Δ1–14-G129R-hPRL were found to behave similarly in all *in vitro* experiments, animal studies were performed using Δ1–9-G129R-hPRL as the prototype of the new generation of antagonists (other analogs were also tested when appropriate). Both the antagonistic effect and the absence of agonism toward the mouse PRLR were assessed in the transcriptional assay involving this receptor (clone ML-F5; Fig. 4).

Concentration of hPRL Analogs in Serum of Treated Mice

Due to the N-terminal deletions, the double mutants Δ1–9-G129R-hPRL and Δ1–14-G129R-hPRL failed to be recognized by any of the commercially available RIA and ELISA kits that

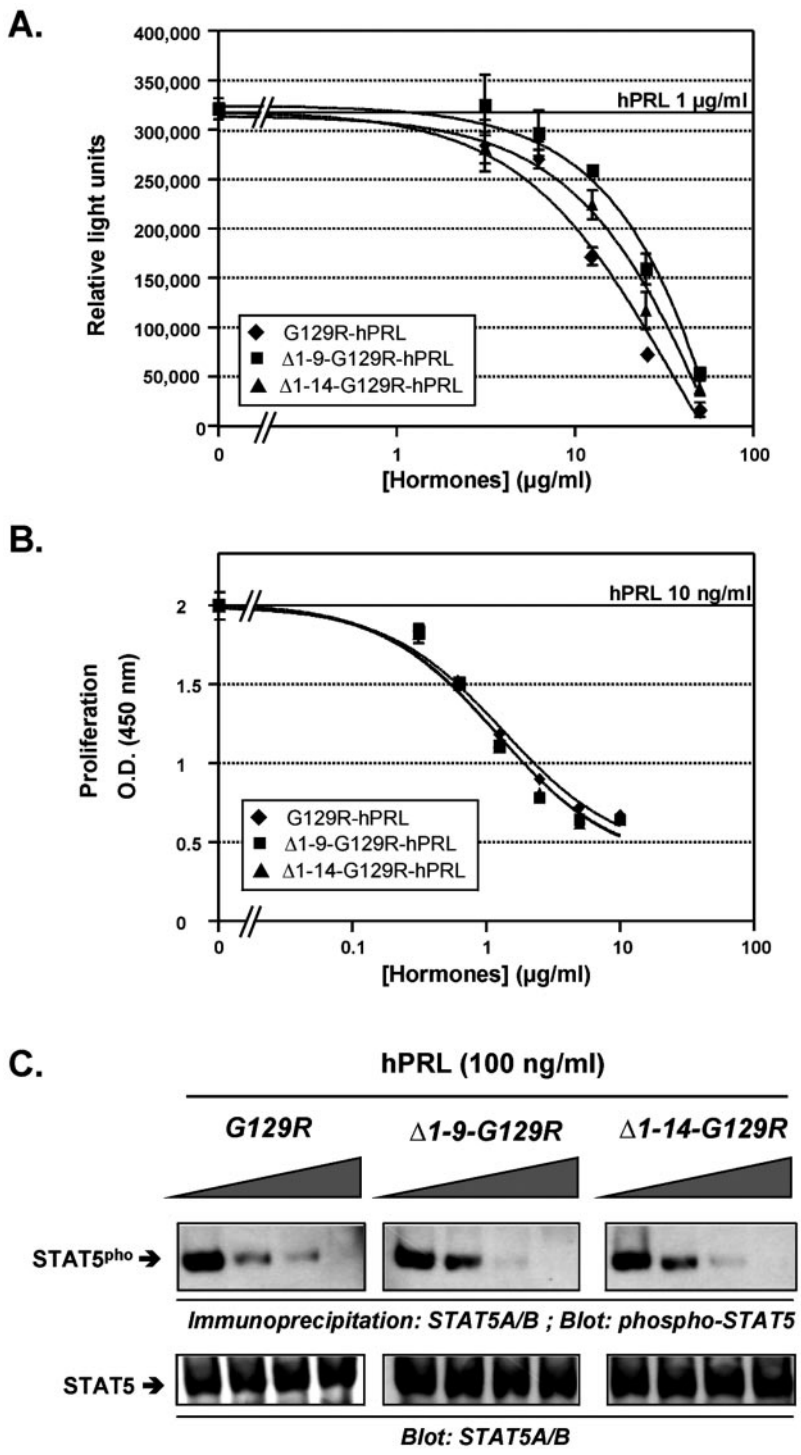

FIG. 2. ***In vitro* antagonistic properties of G129R-containing hPRL analogs.** Antagonistic properties of the three G129R-containing hPRL analogs were assessed based on their ability to compete with a fixed concentration of hPRL (as indicated) for activation of the LHRE-luciferase reporter gene using HL5 clone (*A*), the proliferation of Ba/F-LP cells (*B*), or the activation of STAT5 phosphorylation (immunoprecipitated using appropriate antibodies) in T47D human breast cancer cells (*C*), all assays involving the human PRL receptor. Data are from one experiment representative of at least two independent experiments performed in triplicate. *A* and *B*, *error bars* represent S.D. (when not visible, they are smaller than symbols). These data show that N-terminal deletions do not affect the antagonistic properties reported previously (39) for G129R-hPRL in these assays.

we tested, whereas other hPRL analogs were recognized as expected. In order to quantify serum concentrations of the antagonists injected into mice, we modified the PRL ELISA kit, as described under "Experimental Procedures," by substituting a polyclonal antibody for the secondary HRP-conjugated monoclonal anti-hPRL antibody (which we showed to map the N-terminal epitope of hPRL). Specific standard curves using several hPRL analogs produced by recombinant strategy (G129R, Δ1–9-G129R-hPRL, Δ1–9-hPRL, S179D) were then performed to validate this modified configuration of the ELISA. As shown in Fig. 5, all analogs, including N-terminal deleted mutants, could be reliably measured using this modified ELISA procedure. The concentration of all analogs in the serum of treated mice was estimated using the dose-response curves obtained for the same analog, as shown in Fig. 5.

Co-injections of hPRL and Δ1–9-G129R-hPRL in WT Mice

MAPK Activation in the Liver—The ability of the new antagonist Δ1–9-G129R-hPRL to compete the effect of WT hPRL was first assessed by co-injecting various ratios of hPRL *versus* Δ1–9-G129R-hPRL into WT mice. The liver was chosen, because its high content of the short PRLR isoform ensures high responsiveness to hPRL stimulation by MAPK phosphorylation (51). As shown in Fig. 6*A*, Δ1–9-G129R-hPRL is able to inhibit

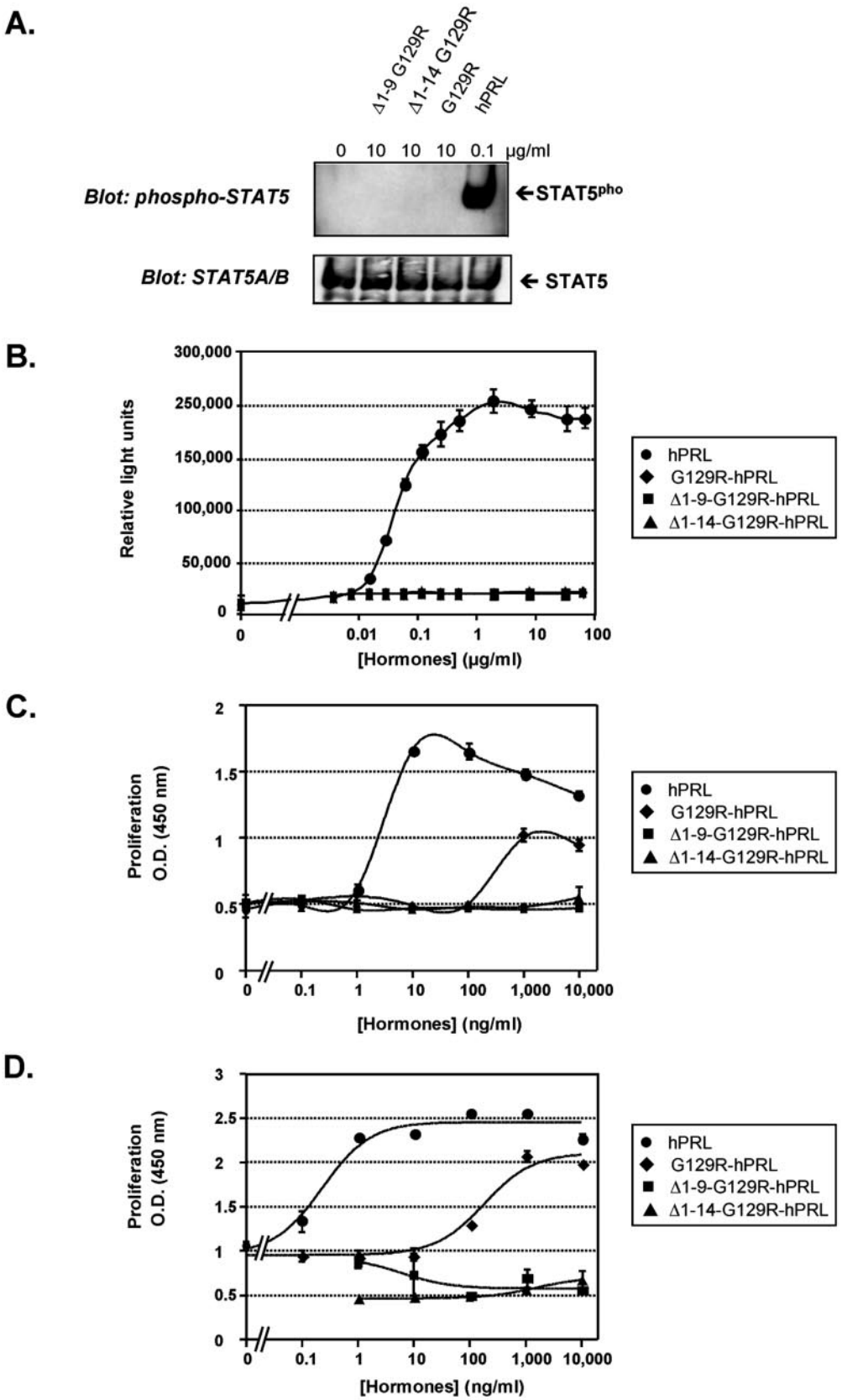

FIG. 3. ***In vitro* agonistic properties of G129R-containing hPRL analogs.** Agonistic properties of the three hPRL analogs were assessed based on their ability to activate STAT5 phosphorylation in T47D human breast cancer cells (*A*), to activate the LHRE-luciferase reporter gene using HL5 clone (*B*), and the proliferation of Ba/F-LP (*C*) or Nb2 (*D*) cells. Data are from one experiment representative of two (*A*) or three (*B–D*) independent experiments performed in triplicate. *Vertical lines* represent S.D. (when not visible, they are smaller than symbols). In less sensitive assays (*A* and *B*), none of the analogs display any agonistic effect (curves of the three G129R-containing analogs are superimposed in *B*). In contrast, whereas G129R-hPRL exhibits mid or submaximal activity in the more sensitive proliferation assays, both N-terminal deleted G129R analogs fail to activate the receptor, even at a minimal level. Based on data shown in Figs. 2 and 3, we suggest Δ1–9-G129R and Δ1–14-G129R hPRL analogs should be considered to be pure antagonists *in vitro*.

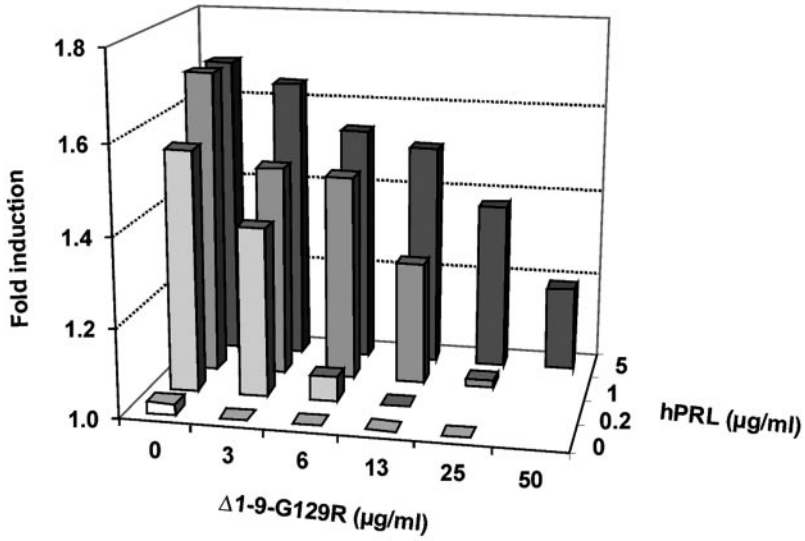

FIG. 4. ***In vitro* biological properties of Δ1–9-G129R-hPRL toward the mouse PRL receptor.** Before performing *in vivo* assays in mice, the biological properties of Δ1–9-G129R-hPRL were assessed in cells expressing the mouse receptor. To that end, a stable clone (ML-F5) containing the LHRE-luciferase plasmid and the expression vector encoding the mouse PRLR was generated. Although the maximal effect of PRL in this bioassay was much lower than for the HL5 clone (~2-fold induction), this experiment clearly demonstrates that Δ1–9-G129R does not exhibit any agonistic activity toward this receptor (*bars* with 0 μg/ml hPRL), whereas it is antagonizes the effect of WT hPRL in a dose-dependent manner (more efficient against 0.2 than 5 μg/ml hPRL).

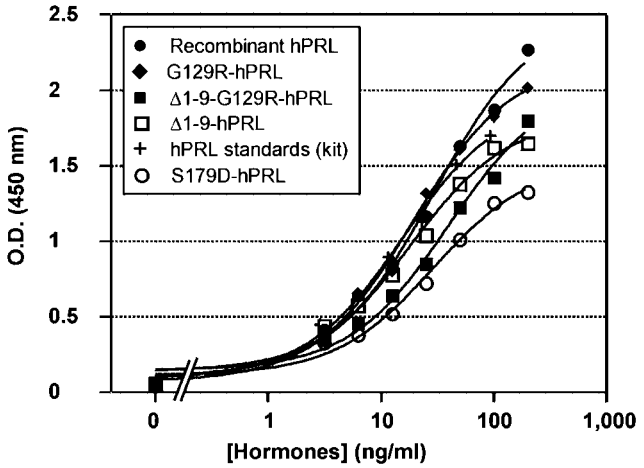

FIG. 5. **Modified hPRL ELISA to quantify N-terminally deleted hPRL analogs.** N-terminally deleted hPRL analogs failed to be recognized using the ELISA kit purchased from Dbc (see "Experimental Procedures"). We then substituted a polyclonal anti-hPRL antibody (A569, Dako) for the original secondary monoclonal anti-hPRL which we showed to map an epitope presumably involving the N terminus. This figure shows that purified N-terminally deleted (Δ1–9), G129R-containing hPRL analogs are recognized in this modified version of the hPRL ELISA almost as well as our recombinant WT hPRL or the hPRL standards provided in the kit, whereas rat and mouse PRL were not (data not shown). Such dose-response curves were used as references for quantifying serum concentrations of G129R-hPRL and Δ1–9-G129R-hPRL in animal studies.

PRL-induced activation of this pathway at 1:50 molar excess, whereas the single mutant G129R does not do so even at a 1:100 ratio. This confirms that Δ1–9-G129R-hPRL is a pure antagonist, whereas the intrinsic agonistic activity of G129R-hPRL predominates over antagonism under these conditions.

Activation of STATs in the Mammary Gland—The responsiveness of the mammary gland to PRL requires the priming effect of hormonal changes that occur during gestation. However, due to the extremely high PRL levels observed in gestation/lactation (up to hundreds of ng/ml), the antagonistic properties of PRL analogs were barely detectable under these conditions (data no shown). Therefore, pregnant WT female mice were first treated with bromocriptine to lower systemic PRL levels, and then the animals were injected with exogenous WT PRL, alone or combined with the antagonists, which in addition, also allows monitoring the antagonist:WT PRL ratio (Fig. 6*B*). As monitored by anti-phosphorylated STAT Western blots, activation of both Stat5 and Stat3, two targets of hPRL in mammary cells (34), was significantly inhibited by co-injection of Δ1–9-G129R-hPRL analog (>50 and >70% inhibition, respectively, as quantified by densitometric analysis of autoradiographies).

Injections in Probasin-rPRL Transgenic Mice

MAPK Activation in the Prostate of Probasin-PRL Transgenic Mice—Local or systemic overexpression of PRL in transgenic mice leads to prostate hyperplasia (44, 52). At the molecular level, we showed that this phenotype parallels the constitutive activation of MAP kinases Erk1 and Erk2 (Fig. 7). Interestingly, transgenic mice expressing the G129R-hPRL analog also show constitutive activation of MAPKs, although clearly at a lower level than observed in PRL transgenic animals, which correlates with the partial agonistic activity of this analog.

To assess the ability of Δ1–9-G129R-hPRL to inhibit the effects of autocrine PRL, we used probasin-rPRL transgenic mice that specifically express rat PRL in the prostate, leading to the hyperplastic phenotype. Injection of Δ1–9-G129R-hPRL was able to revert MAPK activation in a dose-dependent manner (Fig. 8), with maximal effect obtained at a dose of 1 mg/animal (35–45 g) leading to a circulating level of the antagonist in the range of 35–45 μg/ml as estimated by ELISA. Interestingly, the G129R-hPLR analog was less able to compete with autocrine PRL under exactly the same conditions, again in good agreement with its partial intrinsic agonistic activity *in vivo*.

Activation of STATs in the Prostate—The epithelial defects reported in the prostate of STAT5 knockout mice (53) prompted us to analyze STAT5 activation in the prostate of probasin-PRL transgenic mice. This was performed using a new technology based on the detection of activated STAT5 by specific antibodies in an ELISA-like assay, which presents the advantage of requiring very low amounts of protein lysates (5–10 μg per well) in comparison to immunoprecipitation experiments (~1 mg of protein). STAT5 was found to be constitutively activated in transgenic animals (Fig. 9) in comparison to WT littermates. After 1 h of treatment with the new antagonist, the level of STAT5 activation returned to basal levels at the higher dose injected (1 mg). Under the same conditions, G129R-hPRL had only a partial inhibitory effect, and the S179D-hPRL analog failed to show any antagonistic effect. We were unable to detect STAT3 activation using a similar assay. One possible explana-

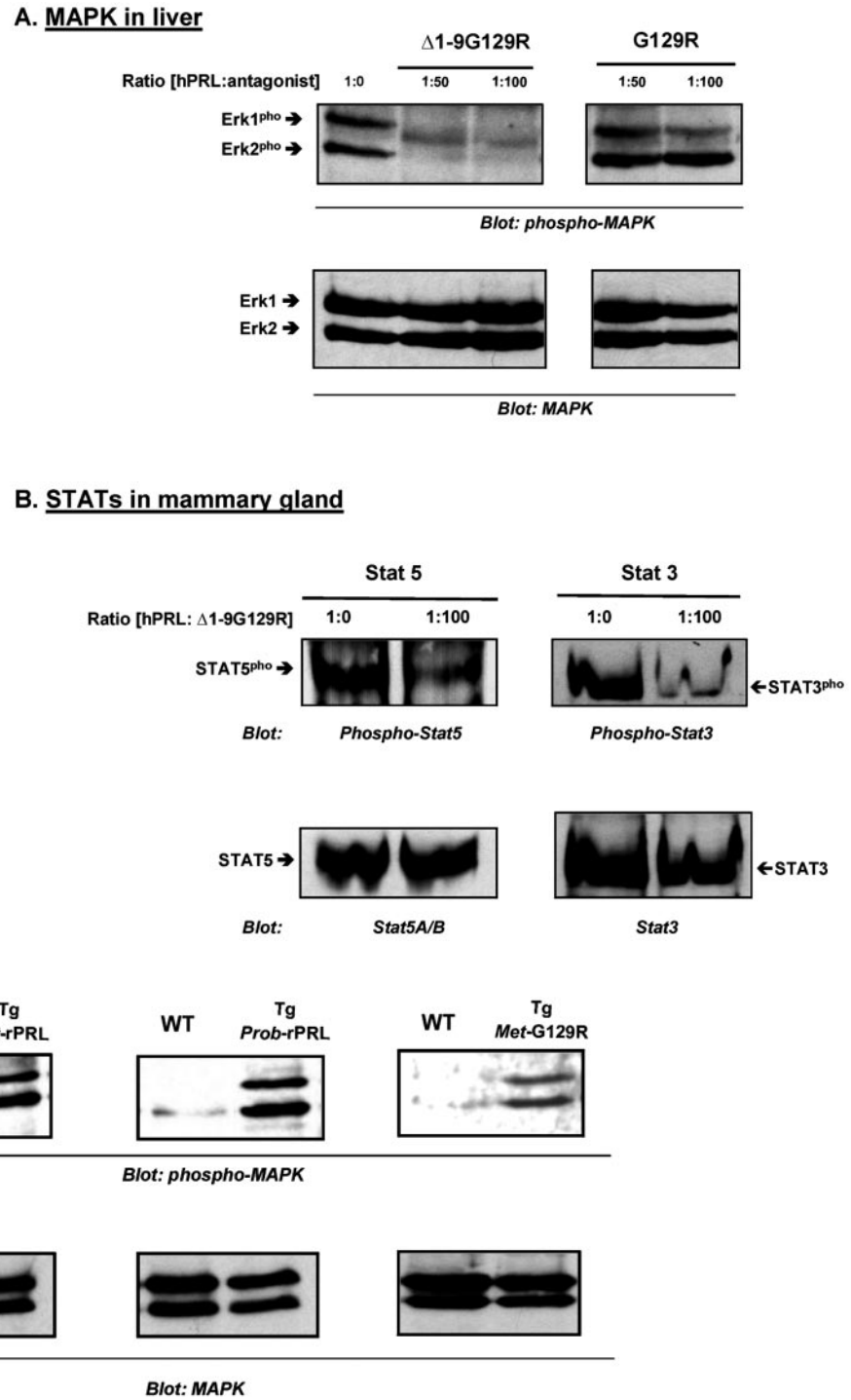

FIG. 6. ***In vivo* antagonistic properties of Δ1–9-G129R-hPRL against exogenous hPRL.** Eight-week-old wild type Balb-c/J females were treated with 10 μg of hPRL or different ratios of hPRL *versus* antagonist (G129R-hPRL or Δ1–9-G129R-hPRL) as indicated. *A* shows the level of MAPK phosphorylation in the liver after a 60-min treatment. Although a 50-fold molar excess of Δ1–9-G129R-hPRL inhibits MAPK activation, G129R fails to antagonize WT hPRL under the same conditions. *B* shows that Δ1–9-G129R-hPRL is also able to compete with co-injected WT hPRL for the activation of STAT3 and STAT5 (immunoprecipitated with appropriate antibodies) in lactating mammary gland after a 30-min treatment (see "Experimental Procedures" for details).

FIG. 7. **Constitutive activation of MAPK in the dorsolateral prostate of PRL and G129R-hPRL transgenic mice.** Transgenic mice expressing rat PRL systemically (metallothionein promoter (*Met*)) or specifically in the prostate (probasin promoter (*Prob*)) develop prostate hyperplasia (see text). Transgenic mice overexpressing G129R-hPRL under the control of metallothionein promoter (line 114) also exhibit a prostate phenotype reminiscent of PRL transgenic animals. This figure shows that in these hyperplastic tissues, MAP kinases are constitutively activated in dorsolateral lobes (as well as in ventral lobes, data not shown), clearly demonstrating that G129R-hPRL exerts significant agonistic effect *in vivo* (WT littermates are shown as controls). Anti-MAPK immunoblots are shown below anti-activated MAPK blots to assess equal protein loading.

tion is that the STAT3 antibodies appeared to be less active in this assay configuration.[3]

DISCUSSION

There are currently three human PRLR antagonists that have been reported. Chronologically, the first to be developed was the hGH analog G120K-hGH, which was shown to be a potent antagonist of the hGHR (30) and subsequently of the hPRLR (33, 54). The first PRLR antagonist based on the hPRL molecule was the prototype first generation antagonist, G129R-hPRL, that we developed based on the same rationale as the hGH-G120K analog, *i.e.* by sterically hindering the second binding site (31, 32). The third, developed 5 years ago, is S179D-hPRL, a mutant designed to mimic phosphorylated

[3] V. Mainfroid, Eppendorf Array Technologies, personal communication.

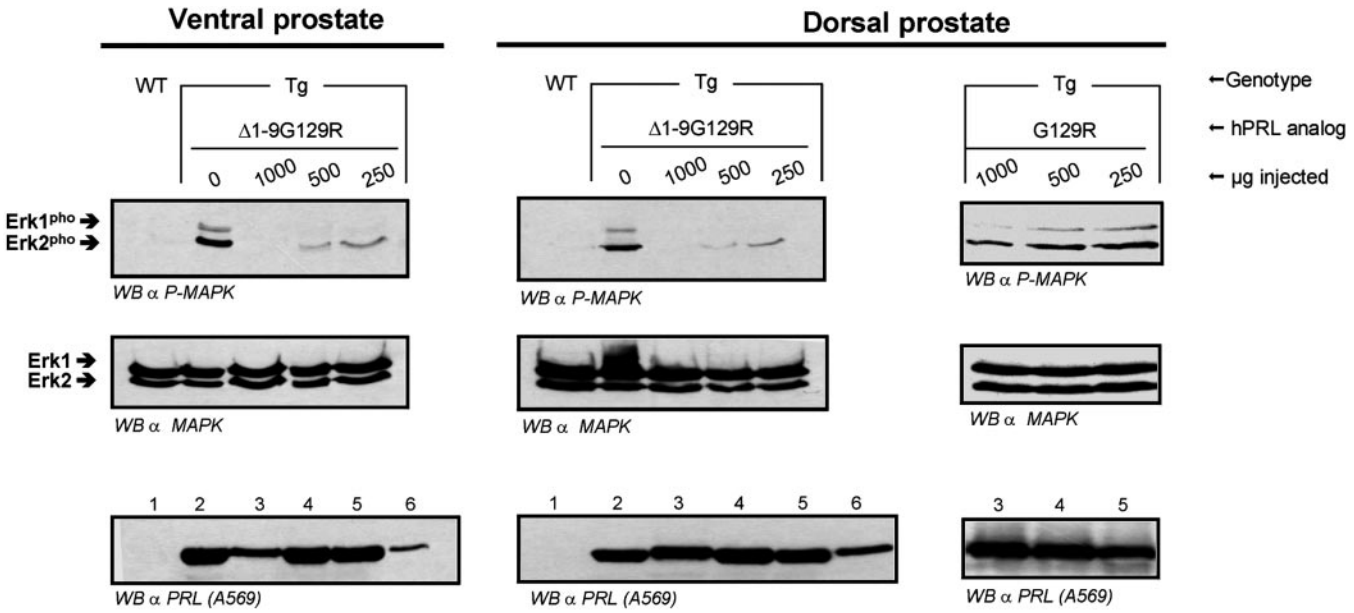

FIG. 8. **Δ1–9-G129R-hPRL inhibits the autocrine/paracrine effect of rat PRL on MAPK activation in the prostate of probasin-PRL transgenic mice more efficiently than G129R-hPRL.** For MAPK immunoblots, the antagonistic properties of G129R-containing hPRL analogs were assessed based on their ability to compete with locally produced rat PRL for MAPK activation in ventral and dorsolateral prostate lobes. A clear decrease of MAPK activation is observed as a function of the dose of Δ1–9-G129R-hPRL injected in probasin-PRL transgenic mice, with total inhibition at 1 mg of the analog. In contrast, the inhibitory effect of G129R-hPRL, if any, is very weak. Control anti-MAPK immunoblots are shown to demonstrate equal protein loading. For PRL immunoblots, membranes were stripped and reprobed using polyclonal anti-hPRL antibodies (A569, Dako), which recognized rat PRL (shown in prostate lysates of untreated mice; *lane 2*) and Δ1–9-G129R-hPRL (20 ng of purified recombinant PRL; *lane 6*). Because both PRLs have similar molecular mass (22 kDa) and electrophoretic mobility, they are undistinguishable in prostate samples from animals treated with the antagonist (*lanes 3–5*).

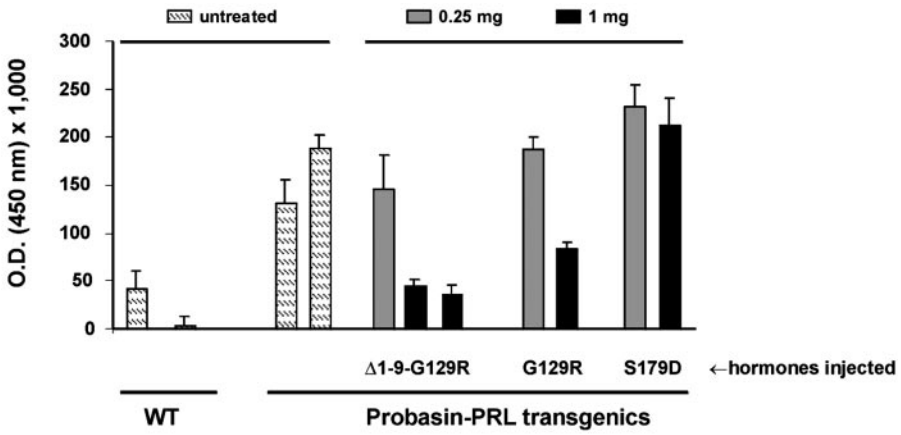

FIG. 9. **Δ1–9-G129R-hPRL inhibits the autocrine/paracrine effect of rat PRL on STAT5 activation in the prostate of probasin-PRL transgenic mice more efficiently than G129R-hPRL.** The antagonistic properties of various hPRL analogs were assessed based on their ability to compete with locally produced rat PRL for STAT5 activation in dorsolateral prostate lobes of probasin-PRL transgenic mice (injection experiments). This was shown using the "TransAM STAT" kit (see "Experimental Procedures"). Each *bar* corresponds to one animal, and *vertical lines* are S.D. of triplicate measurements of each sample. The level of STAT5 activation is more elevated in PRL transgenic mice compared with WT animals. Treatment with 0.25 or 1 mg of Δ1–9-G129R-hPRL clearly inhibits STAT5 activation in a dose-dependent manner. G129R-hPRL partly inhibits STAT5 activation at the highest dose (1 mg), although it was less efficient than Δ1–9-G129R-hPRL. In contrast, the S179D-hPRL analog failed to have any antagonistic effect on this parameter.

hPRL (55). Although a large body of literature describes the antagonistic properties of these three analogs in various *in vitro* bioassays, they all present one or more disadvantages in view of their putative clinical use for cancer therapy. G120K-hGH appears to antagonize two different receptors, namely the hGHR and the hPRLR (30, 54), which is pharmacologically a limiting factor with respect to target specificity. In addition, its ability to inhibit efficiently the PRLR-mediated signaling *in vivo* has been questioned in at least one report, suggesting that it may actually activate, rather than antagonize, the PRLR in rats (56). In addition, mice transgenic for G120R-hGH were not reported to be sterile (57), which is reminiscent of G129R-hPRL transgenics and strongly suggests that, at least in the mouse, these analogs are unable to abolish completely PRLR-mediated signals. The third putative antagonist, S179D-hPRL, is even more controversial. Although some reports indicate that this analog is able to inhibit certain actions of PRL (58), in our hands, it acts as a true agonist, sometimes even as a super-antagonist, but never as an antagonist (42). This finding was again confirmed in the present study. Recent data from Walker and co-workers (59) showed that this analog activates the PRLR and results in β-casein expression, which confirms that,

at least under some instances, S179D-hPRL can mimic or even function better than WT hPRL. Taken together, these experimental observations indicate that there is a need to develop pure PRLR antagonists, because the three molecules currently available all exhibit some level of agonism, which obviously prevents their use at high concentrations necessary to efficiently compete with endogenous PRL in pathological states such as breast or prostate cancer.

We have recently shown that the greater the difference in the affinity of each binding site (site 1 higher), the lower the agonistic activity of the hormone (46). Therefore, our efforts to produce pure PRLR antagonists were focused on testing various strategies aimed at increasing site 1 affinity and/or decreasing site 2 affinity to abolish the undesired residual agonistic activity of G129R-hPRL. However, none of these strategies were successful. For example, the design of a zinc coordination site within the hPRL site 1 homologous to the natural zinc coordination site found in hGH site 1 (60) was shown to be intrinsically detrimental to the affinity of hPRL for its receptor (46). Similarly, introducing within the G129R-hPRL various combinations of mutations enhancing site 1 affinity (43) or decreasing site 2 affinity (A22W mutation) (32) failed to achieve the expected improvement of this antagonist.[2]

In the course of a classical structure-function study aimed at characterizing the functional involvement of the N-terminal tail of hPRL, which is the region within the PRL/GH hormone family with the greatest sequence difference (27), we actually found that deletion of the first 9 amino acids slightly increases hormone activity, an effect presumably mediated by site 1 enhancement, whereas deletion of residues 1–14 decreases activity, presumably by affecting site 2 affinity (68). Thus, these N-terminal deletions were inserted into the G129R-hPRL analog because they were anticipated to improve the antagonistic properties of the latter either by increasing its site 1 affinity or by altering that of site 2. Unexpectedly, despite the fact that both N-terminal deletions (Δ1–9 and Δ1–14) have opposite effects on hormone affinity and bioactivity (68), the double mutants (Δ1–9-G129R-hPRL and Δ1–14-G129R-hPRL) displayed the same 10-fold reduced affinity for the human receptor compared with WT hPRL. With respect to biological properties, the results obtained for these new antagonists mutants are also superimposable. Whatever the bioassay employed, even using the highly sensitive Ba/F-LP or Nb2 proliferation assays, both new analogs failed to stimulate the receptor, even at a minimal level. This is in sharp contrast to the currently available PRLR antagonists (G120R-hGH, S179D-hPRL, and G129R-hPRL), which all exhibit significant agonism in at least one of these assays (31, 39, 42, 50). Thus, although the new mutants do not provide any significant improvement with respect to overall affinity, and thus to their IC_{50} values in antagonistic studies, the absence of residual agonism confers to these new compounds the advantage of acting as pure antagonists.

The observations of *in vitro* assays were all confirmed *in vivo*. In animal studies involving treatment with high doses of the hormones of interest, the new antagonist Δ1–9-G129R-hPRL never exhibited any detectable agonism. In addition, it abolished all PRL-mediated signals investigated, irrespective whether competition was exerted against exogenous (co-injection experiments) or autocrine (probasin-rPRL mouse studies) PRL. This latter observation provides evidence that these new pure antagonists are good candidates to counteract the proliferative effects induced by locally produced PRL, because constitutive MAPK or STAT5 activation reflects autocrine-paracrine action of PRL leading to prostate hyperplasia (44). Under identical conditions, G129R-hPRL failed to efficiently inhibit PRLR-mediated activation of these signaling cascades. Because both G129R-containing antagonists exhibit similar affinity and were present at similar concentrations in serum of treated mice as estimated by ELISA, the partial inhibitory effect of G129R-hPRL is not likely due to an insufficient concentration, but rather reflects an intrinsic residual agonism, which causes low level activation at high amounts. This contrasts with a recent report by Chen *et al.* (38) claiming that G129R-hPRL antagonizes tumor growth-promoting effects of PRL *in vivo*. Our recent studies (39) clearly showed that PRL analogs can shift from antagonists to partial agonists depending on the parameter analyzed and of assay sensitivity. Therefore, we cannot exclude that the assay used by Chen *et al.* (38) exhibits the characteristics of low sensitivity bioassays, allowing G129R-hPRL to exert some antagonism. This correlates with the fact that in their study, mammary tumor cell growth was seen in animals primed with estrogen, which itself is a potent mitogen able to induce tumor proliferation, perhaps explaining the partial inhibitory effect of G129R-hPRL. Although the antagonistic activity of G129R-hPRL has proven to be versatile depending on the bioassay or the parameter studied (31, 32, 39, 46, 50), the observation that G129R-hPRL transgenic mice exhibit minimal phenotypes, resembling moderate hyperprolactinemic states (PRL transgenic mice; Fig. 7), ultimately confirms the intrinsic agonistic potency of this analog *in vivo*, thus preventing its use as an effective antagonist for experimental as well as for clinical purposes.

Because the single G129R substitution results in 10-fold lower affinity for still not fully understood reasons, second generation agonists must be present in molar excess (*versus* WT PRL) to exert efficient antagonism. As expected, 50-fold molar excess of Δ1–9-G129R-hPRL completely abolished the effects of WT PRL in co-injection experiments, which is in total agreement with our previous reports (32, 34, 39) using G129R-hPRL in bioassays that allow detection of antagonism. When competing with locally produced PRL, we observed that injection of 0.25 to 1 mg of Δ1–9-G129R-hPRL was able to inhibit constitutive MAPK activation in the prostate of probasin-PRL transgenic mice by >90%. Under these conditions, the circulating concentration of antagonists was around 35–45 μg/ml, which is clearly rather excessive to be able to claim that these new molecules are potent PRLR inhibitors. This result deserves further comments. First, the active doses of antagonists used in this study (0.25–1 mg/animal, which corresponds to ~6–25 mg/kg) are not that much different from the doses of long acting formulation of hGH antagonist (B2036-PEG) required to down-regulate insulin-like growth factor-1 levels in mice (5–10 mg/kg/day) (61). Second, because in probasin-PRL transgenic mice, the local concentration of autocrine PRL cannot be quantified in the prostate (it is not detected in serum) (44), the actual molar excess of the antagonist *versus* local PRL cannot be determined. Western blotting of prostate homogenates with PRL antibodies (Fig. 8) suggests that WT PRL and the antagonist are present at similar concentrations within the tissue (compare *lanes 2* with *lanes 3–5*), although this approach is not really quantitative. This suggests either that expression of the transgene leads to a very high local concentration of PRL or that the high concentration of the antagonist in serum is not that actually found within prostate tissue. Finally, the high concentration of PRL antagonist required to compete local hormone is in the same range as that reported in previous studies involving GH antagonists. For example, although B2036 antagonist efficiently competes with WT hGH at a 1:1 ratio when both ligands are added to cell cultures in an exogenous manner, a molar excess of 6,000-fold of this antagonist is required to inhibit the action of hGH secreted by transfected MCF-7 cells,

i.e. when autocrine hormone must be competed (62). This clearly demonstrates that it is much more difficult to inhibit the actions of autocrine (locally produced) than endocrine (circulating) hormones.

Due to the relatively short half-life (15–20 min) of PRL and GH *in vivo* (63), analysis of long term effect of the antagonists on prostate gene expression was performed by implanting osmotic mini-pumps in probasin-rPRL animals to ensure delivery of the antagonists (G129R-hPRL or Δ1–9-G129R-hPRL) at a constant rate over several days. Unfortunately, this approach only allowed an antagonist concentration of ~60–70 ng/ml in serum, which is far from the concentration required to achieve efficient antagonism as shown above. Despite the limits of this approach, DNA chip analyses were performed as described previously (64–66), and they clearly highlighted that first and second generation antagonists give different results, because G129R-hPRL resulted in slight but uniform up-regulation of gene expression, confirming its intrinsic agonistic activity, whereas Δ1–9-G129R-hPRL had the opposite effect, suggesting some antagonism (data not shown). Obviously, these experiments will have to be repeated using either long acting formulation of the antagonist (67) or, perhaps even better, double transgenic mice overexpressing both WT PRL and first or second generation antagonists.

In summary, we have generated second generation antagonists, the prototype of which is Δ1–9-G129R-hPRL, which are completely devoid of the undesired residual agonistic activity detrimental to first generation antagonists (G129R-hPRL). Acting as pure antagonists, these new compounds are good candidates for the inhibition of PRL actions *in vivo*, with particular emphasis on models involving autocrine PRL, for which there is currently no known negative regulator of expression. Long term effects of these antagonists have yet to be assessed, *e.g.* by analyzing the proliferation rate of tumor cell lines stably transfected with plasmids encoding these new hPRL analogs or by generating transgenic mice expressing a high level of antagonist. One of the disadvantages of first generation antagonists is the residual agonistic activity. The development of second generation antagonists has solved this problem. Our next challenge is to prepare third generation antagonists with elevated affinities for the PRLR and an increased half-life.

Acknowledgments—We gratefully acknowledge the help of Michelle Lion in the purification of hPRL analogs and Christine Trivin for the RIA assays. We also acknowledge Dr. Jon Kindblom and Dr. Catherine Rougeot for their helpful advice in prostate dissections; Dr. Dominique Madern for circular dichroism analysis of hPRL analogs; and Dr. Bruce Kelder for his involvement in generating G129R-hPRL transgenic mice.

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