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S179D-Human PRL, a Pseudophosphorylated Human PRL Analog, Is an Agonist and Not an Antagonist

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For many years, our group has been involved in the development of human PRL antagonists. In two recent publications, S179D-human PRL, a human PRL analog designed to mimic a putative S179-phosphorylated human PRL, was reported to be a highly potent antagonist of human PRL-induced proliferation and signaling in rat Nb2 cells. We prepared this analog with the aim of testing it in various bioassays involving the homologous, human PRL receptor. In our hands, S179D-human PRL was able to stimulate 1) the proliferation of rat Nb2 cells and of human mammary tumor epithelial cells (T-47D), 2) transcriptional activation of the lactogenic hormone response element-luciferase reporter gene, and 3) activation of the Janus kinase/signal transducer and activator of transcription and MAPK pathways. Using the previously characterized antagonist G129R-human PRL as a control, we failed to observe any evidence for antagonism of S179D-human PRL toward any of the human PRL-induced effects analyzed, including cell proliferation, transcriptional activation, and signaling. In conclusion, our data argue that S179D-human PRL is an agonist displaying slightly reduced affinity and activity due to local alteration of receptor binding site 1, and that the antagonistic properties previously attributed to S179D-human PRL cannot be confirmed in any of the assays analyzed in this study. (*Endocrinology* 142: 3950–3963, 2001)

PRL IS a pituitary hormone involved in a wide spectrum of biological activities (1), the majority of which are related to lactation and reproduction, as emphasized by the phenotype of mice in which the genes encoding either the hormone (2) or its receptor (3) have been invalidated. The PRL receptor (PRLR) is a member of the cytokine receptor superfamily (4) that closely resembles the receptor of GH (5). As is true for the GH receptor, the PRLR is activated by ligand-induced homodimerization, which involves the sequential interaction of two regions on the hormone (called binding sites 1 and 2) with two PRLR molecules, leading to the formation of an active trimer (6, 7). Among the numerous signaling pathways triggered by the ligand-bound receptor (1, 8), activation of the Janus kinase 2 (JAK2)/signal transducer and activator of transcription 5 (STAT5) cascade probably represents the most widely distributed hallmark of PRLR signaling (9–11).

PRL exists in several molecular isoforms resulting from various posttranslational modifications, including proteolytic cleavages, glycosylation, and phosphorylation (for a review, see Ref. 12). The occurrence *in vivo* of PRL phosphorylation has been clearly demonstrated in bovine pituitary (13), where serine 90 was identified as the major phosphorylation site of bovine PRL (14). In other species the *in vivo* demonstration of PRL phosphorylation has been less definitive (15, 16), and the physiological relevance of phosphorylation sites identified by *in vitro* incorporation of ^{32}P into PRL (17, 18) remains questionable. In humans, although pituitary-purified human PRL (hPRL) preparations obtained from the NIDDK were shown to contain traces of phosphorylated forms (19, 20), definite identification of phosphorylated amino acid(s) awaits further investigation.

Several reports have shown that phosphorylation alters the biological properties of PRL. For example, phosphorylated bovine PRL displays reduced mitogenic activity in the Nb2 cell proliferation bioassay compared with the nonphosphorylated isoform (21). Accordingly, dephosphorylation of hPRL slightly increases its bioactivity (19, 20). Still more remarkably, phosphorylation of serine 177 confers antagonistic properties to rat PRL (rPRL) in the same Nb2 cell proliferation assay (18, 19). This serine residue is highly conserved among PRLs from different species (22) and is homologous to serine 179 in hPRL. Based on the antagonistic properties demonstrated for phosphorylated rPRL, a molecular mimic of a putative S179-phosphorylated hPRL has been engineered in Walker's laboratory by substituting an aspartate for the wild-type (WT) serine 179, generating the so-called S179D-hPRL analog (20). In a first report these researchers reported that S179D-hPRL strongly antagonizes the mitogenic activity of hPRL on Nb2 cells, thereby extrapolating to the human hormone the functional consequences of Ser^{177} phosphorylation earlier reported for rat PRL (20). Surprisingly, contrary to all previous reports describing the

Abbreviations: hPRL, Human PRL; IPTG, isopropylthiogalactoside; JAK, Janus kinase; LHRE, lactogenic hormone response element; MM, molecular mass; PRLR, PRL receptor; rPRL, rat PRL; STAT, signal transducer and activator of transcription; TBST, Tris-buffered saline-Tween 20; V_{el}, elution volume; WT, wild type.

mechanism of action of PRL or GH antagonists (23–27), S179D-hPRL appeared to antagonize hPRL effects through a noncompetitive inhibition of receptor activation (20). This encouraged Walker and colleagues to investigate the molecular basis of this unusual, although apparently highly potent, mechanism of antagonism. Using the immunoblot approach to investigate activation of the JAK2/STAT5 cascade, these researchers showed that S179D-hPRL strongly activates STAT5 while minimally activating JAK2 (28). Based on these observations, they suggested the involvement of a kinase other than JAK2 in STAT5 activation, and hence, they hypothesized that the antagonistic activity of S179D-hPRL may result from activation of signaling molecules/pathways other than those known to be involved in hPRL signaling (1) and still to be identified.

For many years the development of hPRL antagonists that could be of use in reducing breast cancer progression has been one of our goals (8, 29). In this perspective we have previously engineered several hPRL analogs, one of which (G129R-hPRL) appeared to display competitive antagonistic properties in various PRLR-mediated bioassays (25, 26), including hPRL-induced signaling and proliferation of breast cancer cells (27). However, due to its lower affinity, the analog had to be used at a significant molar excess *vs.* WT hPRL (1:10 to 1:50 ratio) to exhibit significant antagonism, which justified further efforts in developing more potent hPRL antagonists. In this respect the conclusions claimed by Walker *et al.* in their two recent reports (20, 28) appeared of great interest and prompted us to take further advantage of the antagonistic properties of S179D-hPRL. Thus, the present work was initiated with the aim to engineer more potent hPRL antagonist by combining the two point mutations [S179D (20) and G129R (25)] individually conferring antagonism to hPRL analogs. However, we failed to confirm the conclusions of Walker's articles regarding S179D-hPRL properties, as this mutant clearly acts as an agonist, and not an antagonist, on the PRLR. The goal of the present report is to better characterize the biological properties of this analog in various cell bioassays and to discuss why antagonistic properties were erroneously attributed to S179D-hPRL.

Materials and Methods

Cultures and reagents

Culture media, FCS, horse serum, geneticin (G-418), trypsin, and glutamine were purchased from Life Technologies, Inc. (Cergy Pontoise, France). Luciferin and cell lysis buffer were from Promega Corp. (Madison, WI), and luciferase activity was measured in relative light units (Lumat LB 9501, Berthold, Nashua, NH). Iodogen was purchased from Sigma (St. Louis, MO), and carrier-free Na[^{125}I] and ampholytes were obtained from Amersham Pharmacia Biotech (Orsay, France).

Hormones

All of the hormones used in this study were produced by recombinant technology: WT hPRL (30), the binding site 2 analog G129R-hPRL (Gly^{129} replaced with Arg) (31), and the molecular mimic of phosphorylated hPRL, so-called S179D-hPRL (Ser^{179} replaced with Asp). The mutants were produced in *Escherichia coli* BL21(DE3) using the pT7L expression vector (32), then purified as described previously (25, 31–33).

Cells

Rat Nb2 lymphoma cells were obtained from P. W. Gout (Vancouver, Canada). As previously reported (26), HL5 cells are a clone of 293 fibroblastic cells stably transfected with the plasmids encoding the hPRLR (34) and a PRL-responsive reporter gene. The latter contains the sequence encoding the luciferase gene under the control of a six-repeat sequence of the lactogenic hormone response element (LHRE; which is the DNA-binding element of STAT5), followed by the minimal thymidine kinase promoter. T-47D cells (human mammary tumor epithelial cell line) were provided by M. N. Norman (Bristol, UK).

Site-directed mutagenesis

Construction of the S179D-hPRL mutated cDNA was performed by the oligonucleotide-directed mutagenesis method using the Chameleon double stranded, site-directed mutagenesis kit from Stratagene (La Jolla, CA), following the manufacturer's instructions. The pT7L-hPRL expression vector (32) was used as a template for mutagenesis. The sequence of the mutated oligonucleotide was the following (5′→3′ noncoding strand, mutated codon *underlined*): GTC GAT TTT ATG GTC ATC CCT GCG TAG. The selection primer (*Xmn*I restriction site; see manufacturer's instructions) was the following: 5′-CAT CAT TGG AAA ACG CTC TTC GGG GCG-3′. Clones containing the expected mutation were identified by DNA sequencing on the pT7L-hPRL plasmid (32).

Biochemical properties

Production and purification of proteins. Recombinant WT hPRL and hPRL analogs were overexpressed in a 1-liter culture of *E. coli* BL21(DE3) and purified as previously described (32). Briefly, when the OD_{600} of bacterial cultures reached 0.7–0.9, overexpression was induced using 1 mM isopropylthiolgalactoside (IPTG) for 2 (20) or 4 h (32) (OD_{600}, ~2.5 after 4 h). Cell lysis was performed using a cell disintegrator (Basic Z, Cell D, Roquemaure, France). Proteins were overexpressed as insoluble inclusion bodies that were solubilized in 8 M urea (5 min at 55 C, then 2 h at room temperature) and refolded by continuous dialysis (72 h, 4 C) against 50 mM NH_4HCO_3, pH 8. Proteins collected at this step of the protocol were referred to as nonpurified. Alternatively, refolded proteins were concentrated by tangential flow ultrafiltration (500 ml/min flow rate) using a YM10 Miniplate bioconcentrator (Millipore Corp.-Amicon, Bedford, MA). After centrifugation (10 min, 9000 × *g*) of concentrated protein, supernatants were purified by gel filtration chromatography using a high resolution Sephacryl S-200 column (Amersham Pharmacia Biotech) equilibrated in 50 mM NH_4HCO_3 and 150 mM NaCl, pH 8. Fractions corresponding to monomeric hPRL (WT and analogs) were collected, pooled, quantified by the Bradford method (Bio-Rad Laboratories, Inc., Ivry-sur-Seine, France), aliquoted, and stored at −20 C. This final fraction was referred to as purified.

SDS-PAGE and protein quantification. Protein size and purity were assessed using 15% SDS-PAGE under reducing (β-mercaptoethanol) or nonreducing conditions (35). Gels were stained using Coomassie blue. Protein preparations were quantified by Bradford protein assay, using BSA as the reference.

Circular dichroism spectroscopy. A Jobin Yvon CD6 circular dichroism spectropolarimeter with a thermostated sample holder was used. Data were recorded at 25 C using 0.1-cm cells with an interval of 1 nm and an integration time of 5 sec. The sample concentration was 0.4 mg/ml in 25 mM NH_4HCO_3 and 150 mM NaCl, pH 8. Each spectrum is an average of three scans, corrected for buffer. Helicity was calculated as described (36).

Apparent molecular mass. The apparent molecular mass (MM) of WT hPRL and hPRL analogs was estimated using analytical gel filtration chromatography (HR S-200) according to calibration from several MM markers: dextran blue (void volume), BSA (68 kDa), carbonic anhydrase (30 kDa), and myoglobin (17.5 kDa).

Isoelectric focusing. The isoelectric point (pI) of hPRL (WT and mutants) was determined by isoelectrofocusing. Electrophoresis was performed under continuous cooling; electrode solutions were 20 mM acetic acid and 20 mM NaOH. The gel contained polyacrylamide (5.5%), glycerol (10%), and ampholytes (5.5%) in a range of pH 3–10. A prerun of 15 min (200 V) was applied to the gel before sample loading to allow formation of the pH gradient. Ten micrograms of protein diluted in sample buffer (5.5% ampholytes and 10% glycerol) were loaded on the gel, then the run

was performed at 200 V until the visible band corresponding to methyl red (pI 3.75) was focused. Gels were fixed in 20% trichloroacetic acid, then in 40% ethanol/10% acetic acid. Staining was performed using Coomassie blue and destaining in 40% ethanol/10% acetic acid. The pI of hPRL samples were estimated by comparison with pI markers (Amersham Pharmacia Biotech).

Biological properties

Binding studies. Binding affinity of S179D-hPRL was determined using cell homogenates of the HL5 clone (expressing the human PRLR), following the procedure previously described (26). Briefly, hPRL was iodinated using Iodogen (33), 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 cell homogenate protein in the presence of 30,000 cpm [^{125}I]hPRL and increasing concentrations of unlabeled WT hPRL or S179D-hPRL. Results are representative of three independent experiments performed in duplicate. The relative binding affinity of the mutant was calculated as the ratio of its IC_{50} with respect to that of WT hPRL.

In vitro cell bioassays.

Nb2 cell proliferation assay. The reference bioassay for lactogenic hormones is the lactogen-induced proliferation of rat Nb2 lymphoma cells (37, 38). The Nb2 cell line was routinely maintained in RPMI 1640 supplemented with 10% horse serum, 10% heat-inactivated FCS, 2 mM glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, and 100 μM β-mercaptoethanol. The proliferation assay was performed as initially described (37) with minor modifications. Briefly, the assay was performed in 96-well plates using 2×10^4 cells/well on a starting day in a final volume of 200 μl, including hormones. As it will be explained in *Results*, lower cell densities were also tested according to the protocol of Chen *et al.* (20). Cell proliferation was estimated after 3 d of hormonal stimulation by adding 10 μl WST-1 tetrazolium salt (Roche, Meylan, France) as previously described (39). This survival reagent is metabolized by mitochondria of living cells, which leads to an increase in the OD measured at 450 nm (OD_{450}) in a manner that is proportional to the number of cells counted by hemocytometer (data not shown). A minimum of 30 min was required to get homogenous colorimetric reaction, then OD_{450} values increased for the various experimental conditions proportionally to the time of incubation with WST-1. The linearity of OD_{450}/cell density ratios up to absorbance values of 3–3.5 was confirmed by performing preliminary cell dilution experiments (not shown). We report data obtained after 2–4 h of reaction (which fall within this absorbance range) to allow detection of minimal cell proliferation under low stimulation conditions. Antagonistic properties of hPRL mutants were assessed in competition assays using 200 pg/ml or 1 ng/ml WT hPRL. The experiments were performed at least three times in triplicate or quadruplicate.

Human PRLR transcriptional bioassay. The HL5 clone (human PRLR-LHRE-luciferase, clone 5) (26) was routinely cultured in DMEM-Nut F12 medium supplemented with 10% FCS, 2 mM glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, and 700 μg/ml G-418 (clonal selection). The assay was performed in 96-well plates using 5×10^4 cells/100 μl·well in medium containing only 0.5% FCS. Cells were allowed to adhere overnight, then 100 μl hormones diluted in FCS-free medium were added to each well. After 24 h of stimulation, cells were lysed (50 μl), then luciferase activity was counted for 10 sec in 15 μl cell lysate. The absolute values of luciferase activity were found to vary depending on the number of cell passages; however, this did not significantly affect the fold induction of luciferase activity (calculated as the ratio between the relative light units of stimulated *vs.* nonstimulated cells). Maximal fold induction (routinely ~15- to 20-fold) was obtained in the range of 1–10 μg/ml WT hPRL. To avoid interassay variations, all analogs to be compared were systematically tested within each experiment, and data obtained in one experiment representative of three experiments performed in duplicate are shown.

Cell cycle analyses. T-47D cells were routinely cultured in DMEM/Ham's F-12 (1:1) medium containing 10% FCS, 2 mM glutamine, penicillin (50 U/ml), and streptomycin (50 μg/ml). Cells were aliquoted in six-well plates ($0.5–1 \times 10^6$ cells/well) and serum-deprived in 0.5% charcoal-stripped serum for 24 h before addition of hormones. After 24-h stimulation, culture medium was collected to recover floating cells by centrifugation, and attached cells were harvested by brief trypsinization. Cells were pooled and permeabilized using 40 μl DNA-Prep Reagent (Coulter Corp., Miami, FL) before 30-min incubation at 37 C in 0.5 ml DNA intercalculator propidium iodide (DNA-Prep stain propidium iodide). Cell cycle distribution was analyzed by flow cytometry on a FACScan using CellQuest software (Becton Dickinson and Co., Mountain View, CA) and manual gating.

Immunoprecipitation and Western blots. Nb2 cells were starved overnight in FCS-free medium before hormonal stimulation. The next day, cells were pelleted and aliquoted in FCS-free medium at 8×10^7 cells/ml (1 ml/tube) and allowed to equilibrate/recover for 30 min at 37 C before PRL stimulation. Cells were stimulated (5 min at 37 C) using various concentrations of WT hPRL or hPRL analogs, as indicated in the figures. At the end of the stimulation, cells were washed twice with ice-cold stopping buffer as previously described (27), and cell pellets were kept frozen until used. Cells were solubilized in 1 ml lysis buffer (30-min rotation at 4 C) (27). Lysates were centrifuged for 10 min at 13,000 $\times$ *g*, then supernatants were quantified for their protein content by Bradford assay and used for immunoprecipitation. A similar procedure was used for Western blot experiments using T-47D cells, except that cells were stimulated for 30 min by the various hormones.

For immunoprecipitation studies (Nb2 cells), 3 mg total lysate were incubated with polyclonal anti-JAK2 (2.5 μl/ml; Upstate Biotechnology, Inc., Schiltigheim, France) or polyclonal anti-STAT5 (C-17, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), used at 5 μl/ml. After overnight rotation at 4 C, immune complexes were captured using 20 μl protein A-Sepharose slurry (Amersham Pharmacia Biotech) for 1 additional h of rotation at 4 C. Protein A complexes were precipitated by centrifugation, and pellets were washed three times in lysis buffer and boiled in 15 μl 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 T-47D cells using 50–100 μg protein/lane on 10% SDS-PAGE. Electrophoretic transfer onto nitrocellulose membranes (Bio-Rad Laboratories, Inc.) was performed as previously described (27). Membranes were blocked with 5% skimmed milk in Tris-buffered saline-Tween 20 (TBST) for 2 h at room temperature. After washing in TBST, they were incubated overnight (4 C) in 3% BSA/TBST containing 4G10 antiphosphotyrosine antibody (Upstate Biotechnology, Inc.; 1:10,000 dilution) or anti-threonine 202/tyrosine 204-phosphorylated MAPK 1 and 2 (also referred to as antiactive ERK1/2; dilution 1:1,000; New England Biolabs, Inc.). Membranes were again washed in TBST and incubated for 1 h at room temperature with a 1:4,000 dilution of horseradish peroxidase-conjugated antimouse antibody (Amersham Pharmacia Biotech). After washing, immunoblots were revealed by 1-min ECL reaction (Renaissance chemiluminescence kit, NEN Life Science Products, Boston, MA), followed by autoradiography (various exposure times). When required, the membranes were dehybridized as previously described (27). After extensive washing and reblocking, membranes were reprobed using anti-JAK2, anti-STAT5, or anti-MAPK polyclonal antibodies (using antirabbit horseradish peroxidase as secondary antibody). Densitometric analysis of autoradiographies was performed using the image analysis software Scion Image (Scion Corp., Frederick, MD).

Results

Biochemical properties

Protein purification. After solubilization of inclusion bodies, proteins were refolded by continuous dialysis and purified by gel filtration. Figure 1A represents the chromatogram obtained for S179D-hPRL. As previously described for wild-type hPRL (32), the refolded protein is eluted in two major peaks that were analyzed using reducing and nonreducing SDS-PAGE (see *insets* in Fig. 1A). The first peak corresponds to the void volume of the molecular sieve [elution volume (V_{el}), 118 ml] and contains both noncovalent and disulfide-bonded aggregates of S179D-hPRL and most of the contam-

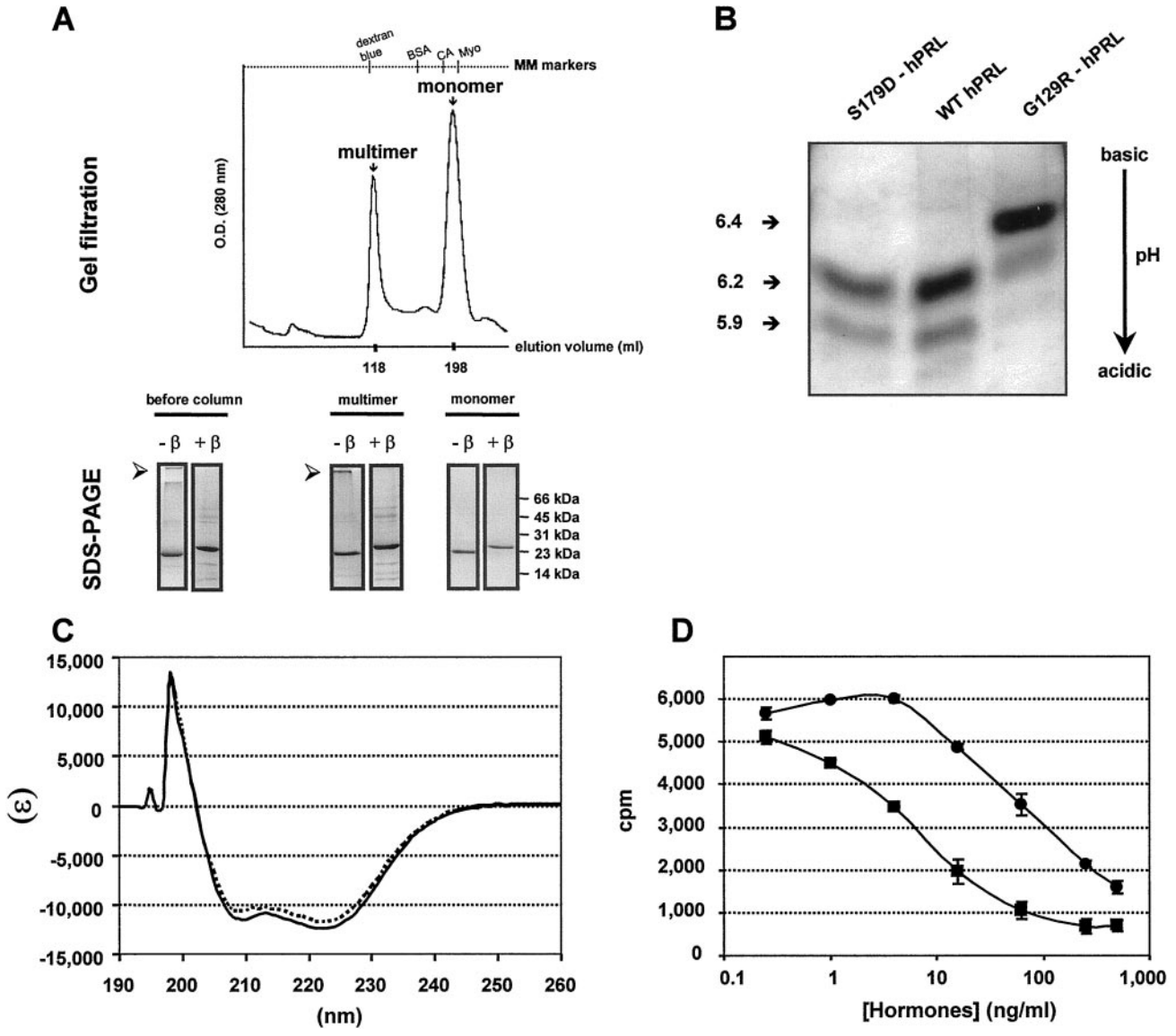

FIG. 1. Biochemical characterization. A, Purification. Renatured S179D-hPRL was purified by gel filtration using a high resolution Sephacryl 200 column (*upper panel*), and each fraction was analyzed by SDS-PAGE in reducing (+β) or nonreducing (−β) conditions (*bottom panels*). Nonpurified S179D-hPRL (before column) contains high MM aggregates that remain at the top of nonreducing gels (*arrowheads*) and disappear in reducing gels, suggesting that they represent disulfide-linked multimers of the mutant. These aggregates are eluted in the first peak of the chromatogram (V_{el}, 118 ml), which contains the multimeric forms of S179D-hPRL and various contaminants. The second peak (V_{el}, 198 ml) elutes between carbonic anhydrase (CA) and myoglobin (Myo) and contains the monomeric form of the analog (>95% pure based on Coomassie blue staining). The difference in S179D-hPRL electrophoretic mobility in reducing and nonreducing conditions is due to the change in the hydrodynamic radius, which is sensitive to the presence or absence of internal disulfide bonds (32). B, Isoelectric focusing. Electrophoresis of WT hPRL or hPRL analogs (S179D or G129R) was performed as indicated in *Materials and Methods*. S179D-hPRL and WT hPRL display the same migration profile, with a major band focusing at pI 6.2 and a minor one at pI 5.9 as indicated. G129R-hPRL focuses in two bands, the major one at pI 6.4 (31). C, Circular dichroism. Protein samples were diluted in 25 mM NH_4HCO_3 and 150 mM NaCl, pH 8, and spectra of WT hPRL (*dotted line*) or S179-hPRL (*continuous line*) were measured in the UV range (190–260 nm). Molar ellipticity (ϵ) is expressed in degree × $cm^2 \times dM^{-1}$. Both spectra exhibit the profile typical of all α-helical proteins, with two minima (208 and 222 nm) and one maximum (195 nm). D, Binding assay. The curves represent competition of [^{125}I]hPRL (30,000 cpm) bound to the hPRLR by serial dilutions of unlabeled WT hPRL (■) or S179D-hPRL analog (●). Each *point* is the average of duplicate measurements calculated from three independent experiments (*error bars* represent the SD). Based on the IC_{50}, S179D-hPRL displays an affinity 20-fold lower than that of WT hPRL.

inants, whereas the second corresponds to the monomeric form of the protein according to column calibration with proteins of known mol wt. Its V_{el} (198 ml) is identical to that of monomeric WT hPRL (23 kDa). Between these two peaks, the chromatogram remains higher than the baseline, reflecting the presence of low mol wt multimers (dimers, trimers, *etc.*). This experiment indicates that refolded, nonpurified S179D-hPRL [as previously reported for WT hPRL (32)] is heterogeneous and contains multiple forms of the protein, which justifies a purification step by gel filtration to isolate monomeric S179D-hPRL.

As Walker and colleagues performed all of their experi-

ments using refolded, nonchromatographically purified S179D-hPRL, we systematically included in all our bioassays both nonpurified and purified (monomeric) fractions of the three proteins used in this study (WT hPRL, S179D-hPRL, and G129R-hPRL). Data obtained with nonpurified hormones are only presented for experiments using Nb2 cells to allow direct comparison with Walker's group results.

Isoelectric focusing. Figure 1B represents a typical isoelectrofocusing experiment of purified WT and hPRL analogs in the pH range of 3–10. As previously described (32), purified hPRL exhibits two bands, the major one focusing at pI 6.2, and the minor one at pI 5.9. According to the addition of a positive charge, the major band of G129R-hPRL focuses at pH 6.4 as reported previously (31). Despite the addition of a negative charge, S179D-hPRL displays the same profile as WT hPRL, with a major band at pI 6.2 and a minor one at pI 5.9.

Circular dichroism. As point mutations can affect regular secondary structures and/or global protein folding, we assessed the α-helical content of S179D-hPRL by circular dichroism as previously reported for other hPRL analogs (31, 33, 40). The profile obtained for S179D-hPRL was almost identical to that obtained with WT hPRL (Fig. 1C), with the two minima at 208 and 222 nm and the maximum at 195 nm typically observed for all α-helix proteins, suggesting no strong (detectable) alteration of the overall content in secondary structures. The calculated helicity was 45% for WT hPRL and 42% for S179D-hPRL.

Biological properties

Binding studies. The affinity of S179D-hPRL for the hPRLR was estimated by its ability to compete with [^{125}I]hPRL for binding to the receptor. Typical competition curves are shown in Fig. 1D. The affinity of WT hPRL, as calculated by Scatchard analysis, indicated a K_d of $3.4 \pm 1.3 \times 10^{-10}$ M. As the competition curves of hPRL and S179D-hPRL are almost parallel in the linear part of the sigmoids (Fig. 1D), the IC_{50} ratio reflects the relative affinity of the mutant for the hPRLR. Averaged from three independent experiments, the IC_{50} values of hPRL and S179D-hPRL were 9.5 ± 1.0 and 181.7 ± 28.9 ng/ml, respectively, indicating a 20-fold reduced affinity of the analog for the homologous receptor. As previously reported (26), the affinity of G129R-hPRL for hPRLR is 10-fold lower than that of hPRL, *i.e.* slightly higher than that of S179D-hPRL.

Bioactivity studies.

Rat Nb2 cells: proliferation assay. We first tested the agonistic properties of S179D-hPRL in the Nb2 assay using the classical procedure recommended by Gout and colleagues (37, 38), who isolated the Nb2 cell clone and established the lactogen-induced proliferation assay. Figure 2A shows cell proliferation induced by increasing concentrations of purified (*left panel*) or nonpurified (*right panel*) WT hPRL, S179D-hPRL, and G129R-hPRL. As expected, monomeric hPRL induces cell proliferation with a maximal effect at about 1 ng/ml, whereas the curve obtained for S179D-hPRL is shifted by 1 log unit toward the higher concentrations. As previously reported (26, 31), the agonistic dose-response curve obtained with G129R-hPRL is shifted by 2 log units compared with that obtained with WT hPRL (Fig. 2A, *left*). It is noteworthy that the two mutants are able to induce a maximal proliferation response after 3 d of stimulation when added at a sufficient concentration; this was confirmed by cell cycle studies performed using FACS analysis (not shown). Although the agonistic parts of the curve obtained for nonpurified WT hPRL and S179D-hPRL (Fig. 2A, *right*) were similar to their purified counterparts, they were more markedly bell-shaped at the higher concentration tested (10 μg/ml). The down-slope in dose-response curves for PRL and GH receptors is thought to reflect a phenomenon of self-antagonism (6, 23). However, as no self-antagonism was observed at the highest concentration with purified proteins (1000 ng/ml in this study, up to 100 μg/ml in Refs. 26, 31, and 33), it is possible that bell-shaped curves obtained using nonpurified hormones could result from an inhibitory effect of contaminants present at high doses of nonpurified preparations (see Fig. 1A and *Discussion*). Finally, nonpurified G129R-hPRL failed to induce proliferation.

We next investigated the antagonistic properties of hPRL analogs. Based on its agonistic properties, S179D-hPRL, whether purified or not, expectedly failed to display any antagonism. Indeed, when increasing amounts of S179D-hPRL were added to 0.2 ng/ml WT hPRL (producing ~50% of maximal cell growth; see Fig. 2A, *left*), an increased dose response of proliferation was obtained from approximately 1 ng/ml of the analog (Fig. 2B, *left*), which appeared to be superimposed on the agonistic curve obtained when testing S179D-hPRL alone (Fig. 2A, *left*). The same observation was made for G129R-hPRL (compare *left panels* of Fig. 2, A and B). These experiments confirm the intrinsic agonistic properties of both hPRL mutants in the Nb2 cell proliferation assay. When 1 ng/ml WT hPRL was competed with nonpurified S179D-hPRL (Fig. 2B, *right*), the diminution of cell growth observed in the higher range of analog concentration was also superimposed on the curve obtained when testing nonpurified S179D-hPRL alone (see Fig. 2A, *right*), indicating that this effect is not a true antagonism, but reflects self-antagonism and/or contaminant effects, as discussed above. On the other hand, the curve obtained with nonpurified G129R-hPRL (Fig. 2B, *right*) may reflect true antagonism, as this protein fraction is intrinsically inactive (Fig. 2A, *right*). Finally, we noticed that in all of the antagonism experiments performed using nonpurified proteins (including WT hPRL), a small decrease in cell response occurred around 0.1–0.2 ng/ml, which may reflect a slight antagonism at this very narrow range of low concentrations, although this was not seen with purified preparations (see *Discussion*).

Our results are in total contradiction with Chen's report (20), which claimed an antagonistic activity for S179D-hPRL. These researchers performed their Nb2 proliferation assays using experimental conditions differing from the classical bioassay protocol, using a lower cell density. Whereas the standard protocol recommends a cell density of 10^5 cells/ml on the starting day (37), Chen *et al.* used 2.5×10^4 cells/ml for agonistic assays and still less (5×10^3 cells/ml) for antagonistic assays. Therefore, we repeated all of our experiments using Chen's protocol. With respect to agonism, we obtained dose-response curves qualitatively similar to those

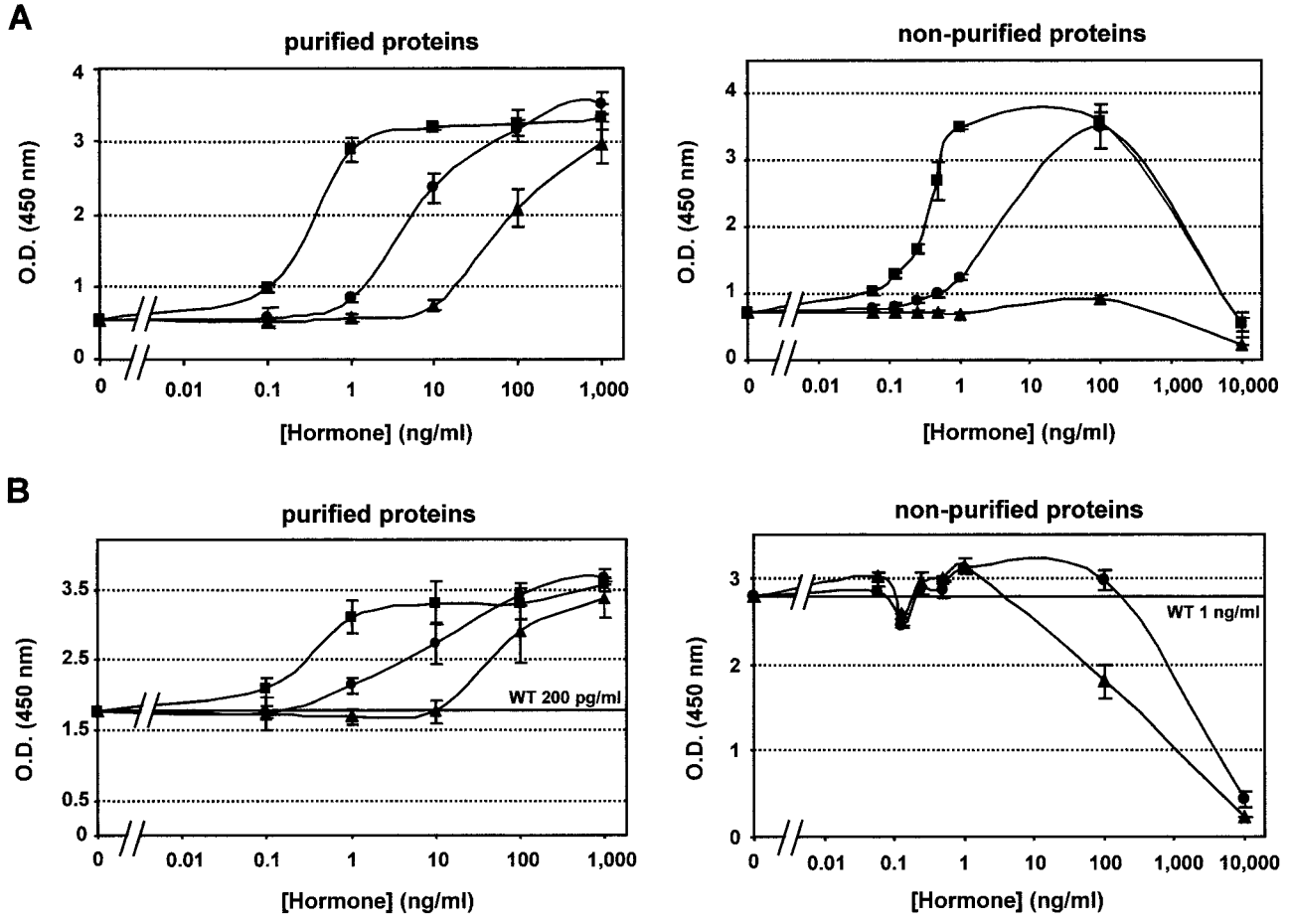

FIG. 2. Nb2 cell proliferation assay. Proliferation assays of Nb2 cells in the presence of purified (*left panels*) or nonpurified (*right panels*) WT hPRL (■), S179D-hPRL (●), or G129R-hPRL (▲) were performed as indicated in *Materials and Methods*, using 10^5 cells/ml on the starting day. Cell proliferation was assessed using WST-1 colorimetric reagent. The data presented are representative of at least three independent experiments that were performed in triplicate or quadruplicate. *Error bars* represent the SD. A, Agonism. Cell proliferation induced by WT hPRL occurs in the range of 0.1–1 ng/ml. Proliferation curves observed in the presence of purified S179D-hPRL or G129R-hPRL are displaced to the high concentration by 1 and 2 log units, respectively, and reach maximal proliferation (same plateau as WT hPRL). Nonpurified WT hPRL and S179D-hPRL lead to bell-shaped curves, whereas nonpurified G129R-hPRL fails to induce cell growth. B, Antagonism. Purified hPRL analogs failed to antagonize cell division induced by 200 pg/ml WT hPRL. The decrease in cell growth in the presence of high concentrations of nonpurified analogs is discussed in the text.

presented in Fig. 2A, except that the changes in OD_{450} values of the colorimetric assay (WST-1 reagent) were lower due to lower cell density (not shown). With respect to antagonistic assays, although using 1000 cells/well (200 μl) led to very small changes in OD_{450} values (<0.1 absorbance unit) that were not very reliable in our hands, we never observed any evidence for S179D-hPRL antagonism (not shown).

In summary, our results clearly establish S179D-hPRL as a weak agonist, and not an antagonist, in the Nb2 cell proliferation assay regardless of the status of protein purification or cell density.

Activation of the JAK/STAT pathway. Based on the observation that S179D-hPRL activates STAT5 without significantly activating the tyrosine kinase JAK2, Walker's (28) group proposed that this analog may exert its antagonistic properties by triggering alternate intracellular signals. As in our hands S179D-hPRL failed to display any antagonistic properties, but clearly acts as an agonist, it was important to confirm its effect on the JAK2/STAT5 pathway in Nb2 cells.

Figure 3 shows dose-dependent activation of JAK2 (A) and STAT5 (B) in Nb2 cells using purified (*left panels*) or nonpurified (*right panels*) hormone preparations. Regardless of the purification status, S179D-hPRL is able to activate JAK2 in a manner similar to WT hPRL, which contrasts to the report of Coss *et al.* (28). Even at 5 ng/ml, a faint band corresponding to phosphorylated JAK2 is already visible, indicating that detection of JAK2 activation does not require stimulation with supraphysiological concentrations of the analog. In contrast, G129R-hPRL failed to induce strong activation of JAK2, even at a concentration of 500 ng/ml (7-fold less phosphorylated JAK2 compared with WT hPRL stimulation; Fig. 3A) or higher (not shown). This clearly indicates that the threshold of activation of signaling pathways does not correlate with that of mitogenic activity (compare Figs. 2 and 3). With respect to STAT5, the same observations were globally drawn (Fig. 5B), as both hPRL and S179D-hPRL (purified) achieved a similar level of STAT5 activation. Again, the level of STAT5 activation was markedly lower using G129R-hPRL, as it was when using nonpurified S179D-hPRL.

As reported previously (28), JAK2 and STAT5 coprecipi-

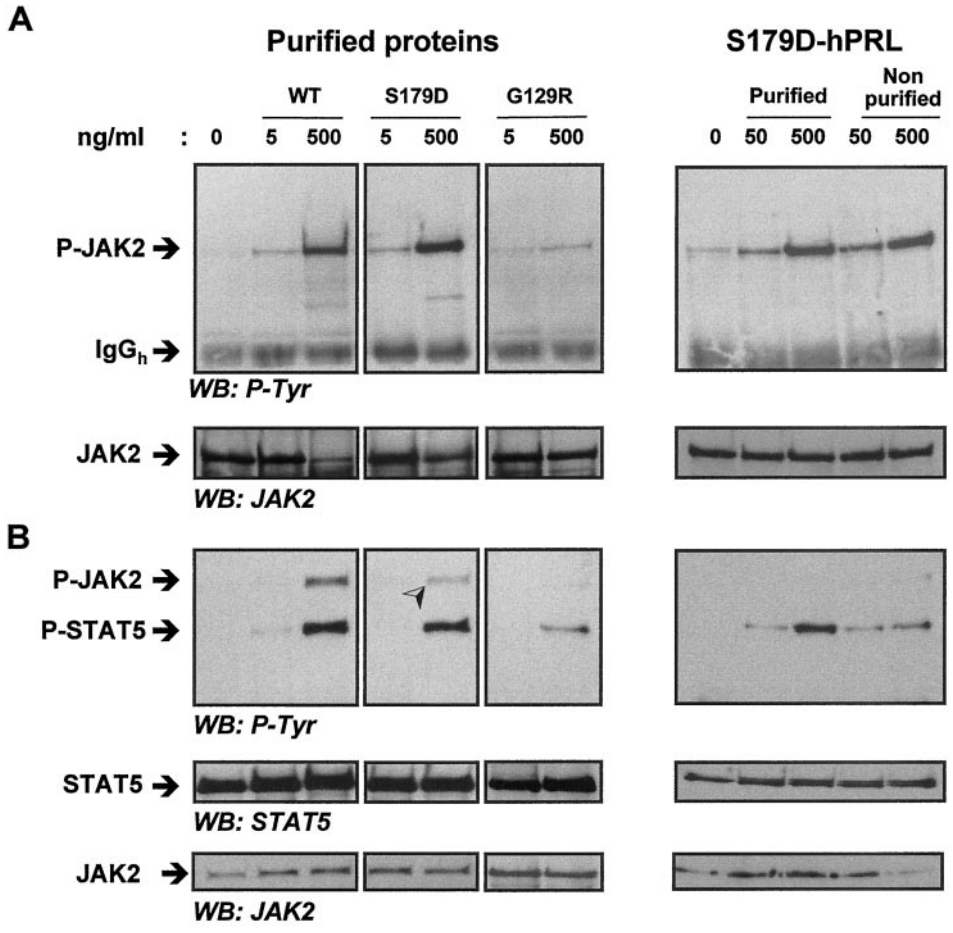

FIG. 3. Agonism of hPRL and hPRL analogs on the JAK2/STAT5 pathway in Nb2 cells. Nb2 cells (8×10^7) were stimulated (5 min) with WT hPRL or hPRL analogs as indicated. Immunoprecipitations (IP) were performed on 3 mg cell lysates using anti-JAK2 (A) or anti-STAT5 (B) antibodies. Immunoprecipitates were analyzed by Western blotting (WB) using antiphosphotyrosine antibody (P-Tyr). Blots were then stripped and reprobed using anti-JAK2 and/or anti-STAT5 antibodies as indicated at the *bottom* of each panel. Whether purified or not, S179D-hPRL is able to induce tyrosine phosphorylation of JAK2 to a similar level as WT hPRL (STAT5 activation was lower using nonpurified S179D-hPRL). In contrast, using identical time exposure for autoradiography, G129R-hPRL fails to activate JAK2 and STAT5 above a minimal threshold. The *arrowhead* indicates the lower level of phosphorylated JAK2 coprecipitating with STAT5 in S179D-hPRL-stimulated cells. IgG_H, Ig heavy chains.

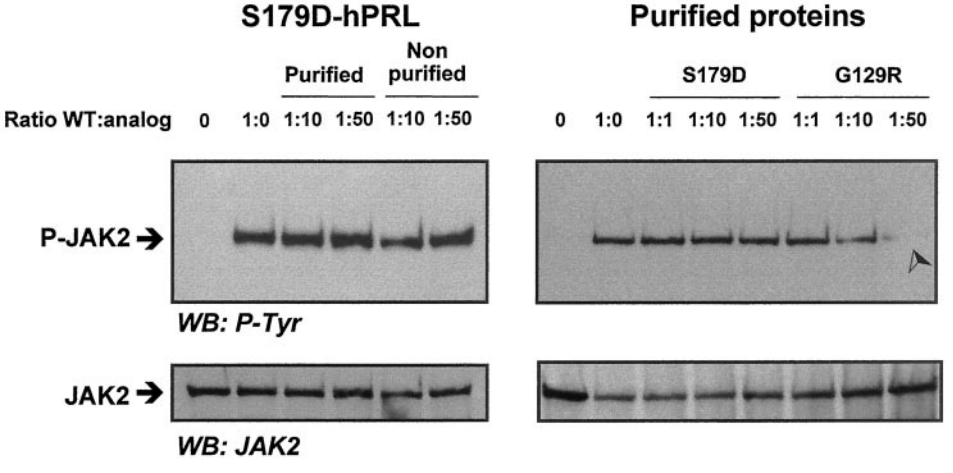

FIG. 4. Antagonism of hPRL and hPRL analogs on JAK2 phosphorylation in Nb2 cells. Cell treatment and immunoprecipitations using anti-JAK2 antibodies were performed as described in Fig. 3 (see also *Materials and Methods*). Antagonism of hPRL analogs was assessed by monitoring tyrosine phosphorylation of JAK2 in the presence of a fixed concentration of WT hPRL (500 ng/ml) *vs.* increasing concentration of analogs (ratio indicated as WT:analog). When added in a 50-fold molar excess, S179D-hPRL failed to alter hPRL-induced JAK2 activation, whereas 10- and 50-fold excesses of G129R-hPRL decreased the level of JAK2 phosphorylation by approximately 1.5- and 8-fold, respectively (*arrowhead*).

tated when using anti-STAT5B antibodies, even in unstimulated cells (*bottom panels* of Fig. 3B). The only difference between WT hPRL and S179D-hPRL that we repeatedly observed in these studies was a lower phosphorylation level of the pool of JAK2 coimmunoprecipitating with STAT5 (*arrowhead* in Fig. 3B). This observation was not analyzed further because it falls out of the scope of this study.

Another finding reported by Coss *et al.* (28) was the ability of S179D-hPRL to competitively inhibit hPRL-induced JAK2 phosphorylation, correlating to some extent with the antagonism observed in the Nb2 proliferation assay by these researchers (20). Using similar approaches, we investigated the antagonistic activity of our S179D-hPRL preparations (purified or not) on hPRL-induced activation of JAK2 (Fig. 4), using G129R-hPRL as a control. S179D-hPRL failed to inhibit the effect of 500 ng/ml hPRL on JAK2 phosphorylation even when added in a 50-fold molar excess, and this was true whether purified or nonpurified analog was used. In contrast, a 50-fold excess of G129R-hPRL clearly competed WT hPRL for JAK2 activation, as the phosphorylation of the

kinase was strongly impaired (by ~10-fold) in the presence of the antagonist (Fig. 4, *right*). The possibility that this inhibition may result from a toxic effect was ruled out, because no cell death was observed over a 3-d proliferation assay using even higher concentrations of this analog (31). It is noteworthy that the antagonistic effect observed on JAK2 was not directly reflected by the level of STAT5 phosphorylation (not shown), which is not fully understood (see *Discussion*).

Human PRLR-mediated bioassays. All of the experiments performed using rat Nb2 cells failed to confirm data previously claimed by others and, rather, supported the conclusion that S179D-hPRL acts as an agonist. Therefore, it was important to assess the properties of this analog in other cell systems, especially in cell bioassays using the homologous (human) PRL receptor. For this purpose we used two bioassays that we recently characterized as good models for highlighting antagonistic properties of human lactogen analogs (26, 27, 39).

Transcriptional activity (HL5 clone). We have previously established a hPRLR-mediated transcriptional bioassay (so-called HL5 clone; see *Materials and Methods*) that enables us to unambiguously identify lactogens displaying antagonistic properties [*e.g.* mutants of hPRL (25, 26) or hGH (39)]. In this assay G129R-hPRL fails to act as an agonist even when tested at extremely high concentrations (120 μg/ml; Fig. 5A). However, as it binds to the receptor it acts as an antagonist (Fig. 5B) (26). In contrast, S179D-hPRL again behaved as a weak agonist, as its curve was slightly displaced toward high concentrations compared with WT hPRL (Fig. 4A). Averaged from three experiments, the ED_{50} of WT hPRL was 2.3 $\pm$ 0.3 μg/ml, whereas a 4-fold higher concentration of the analog (9.7 $\pm$ 0.5 μg/ml) was required to achieve a similar luciferase response. Surprisingly, the maximal activity obtained at the highest concentration tested (120 μg/ml) was systematically higher for purified S179D-hPRL than for WT hPRL (respectively, 23.6 $\pm$ 3.0- and 17.8 $\pm$ 1.8-fold inductions; n = 6; Fig. 5A, *left*). In agreement with its (super) agonistic activity, no evidence of S179D-hPRL antagonism was observed, as highlighted by the dose-dependent increase in luciferase activity when the analog and WT hPRL were tested together (Fig. 5B). These experiments were repeated using nonpurified material, and similar observations were made, *i.e.* that S179D-hPRL activated the hPRLR, whereas G129R-hPRL did not (not shown).

Proliferation of human mammary tumor cells (T-47D). We recently reported that G129R-hPRL was able to competitively inhibit hPRL-induced proliferation of T-47D cells, and this was demonstrated by monitoring both cell density (WST-1) and cell cycle status in time-course experiments (27). The same procedure was repeated here for S179D-hPRL. As expected from the data presented above, this analog stimulated T-47D cell division, as reflected by the FACS profile monitored after 24 h of treatment. Due to its lower affinity for the hPRLR, however, a similar level of cell division required higher amounts of S179D than WT hPRL (Fig. 6 and data not shown). In agreement with our previous report, G129R-hPRL (even at high concentrations) failed to activate T-47D cells. In competition experiments the latter efficiently competed with hPRL for stimulating entry of T-47D cells into the division cycle, whereas S179D-hPRL failed to exert any antagonistic effect.

Stimulation of the MAPK pathway in human mammary tumor cells (T-47D). One of the hallmarks of the antagonistic effect of G129R-hPRL in T-47D cell division is its inhibitory effect on the activation of the MAPK pathway induced by WT hPRL (27). This assay was thus performed to definitely assess the effect of S179D-hPRL on the hPRLR at the molecular level. In agreement with proliferation data, S179D-hPRL did activate ERK1 and ERK2, although a little less efficiently than WT hPRL, whereas G129R-hPRL had no stimulatory effect

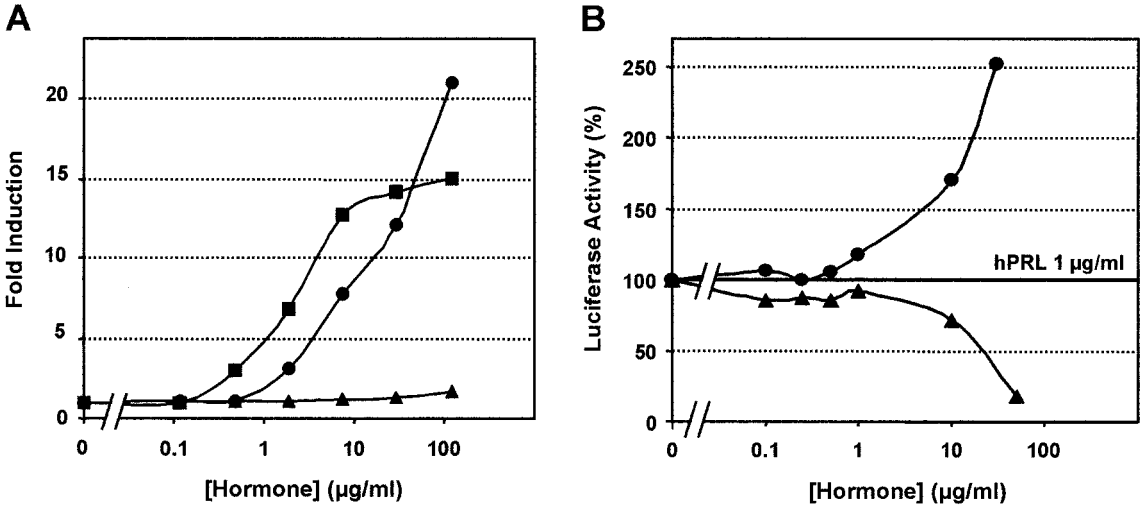

FIG. 5. LHRE-luciferase transcriptional assay. 293 cells stably expressing the hPRLR and the LHRE-luciferase reporter gene (clone HL5) were stimulated for 18–24 h with increasing concentrations of purified WT hPRL (■), S179D-hPRL (●), or G129R-hPRL (▲) as described in *Materials and Methods*. One typical experiment performed in duplicate and representative of at least three experiments is shown. A, Agonism. Although G129R-hPRL is almost inactive, the curve obtained for S179D-hPRL is slightly displaced to the right, but reaches higher final values (125%) compared with WT hPRL. B, Antagonism. Both G129R and S179D hPRL analogs were tested for their ability to compete a fixed concentration of WT hPRL (1 μg/ml, whose luciferase activity was normalized to 100%). G129R-hPRL inhibits the transcriptional activity induced by WT hPRL, whereas S179D-hPRL has an additive effect, reflecting its agonistic activity in this assay (see A).

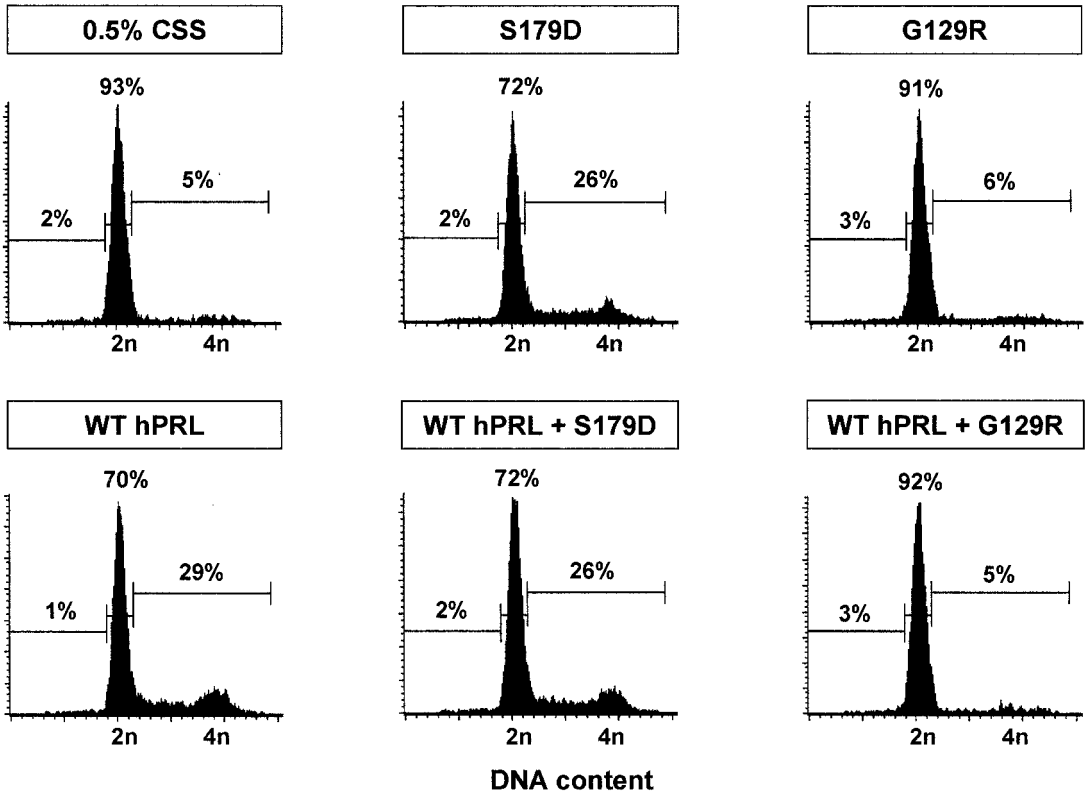

FIG. 6. Cell cycle analyses on human mammary tumor epithelial cells (T-47D). T-47D cells were serum-deprived for 24 h, then incubated in 0.5% charcoal-stripped serum in the absence (control) or presence of hormones, added alone for agonism experiments (100 ng/ml WT hPRL, 10 μg/ml G129R-hPRL, 10 μg/ml S179D-hPRL), or in competition with WT hPRL for antagonism experiments (100 ng/ml WT hPRL combined to 10 μg/ml S179D-hPRL or G129R-hPRL). Cell cycle analyses were performed after 24 h. DNA content (2n and 4n indicated) *vs.* cell number is presented in each panel; from *left* to *right*, *numbers* indicate the percentage of cells in apoptosis (area below 2n label on *x*-axis), in G_0/G_1 (peak centered on 2n label) or in S/M phase (area above 2n label). Note that G129R-hPRL has no effect *per se* compared with the assay medium with respect to cell division or apoptosis (*upper*, *right*), but it completely antagonizes the proliferative effect of hPRL (*lower*, *right*). In contrast, S179D-hPRL stimulates cell division (*upper*, *middle*) and fails to antagonize WT hPRL in competition experiments (*lower*, *middle*). The results were identical using nonpurified hormones (not shown).

(Fig. 7A) (27). In antagonistic experiments, the level of MAPK activation induced by 100 ng/ml hPRL (leading to mid scale activation of MAPK; see Fig. 7A) was competed by G129R-hPRL in a dose-dependent manner. In contrast, S179D-hPRL had an additive effect, leading to maximal levels of MAPK phosphorylation. These observations clearly indicate that S179D-hPRL exerts agonistic, but absolutely no antagonistic, activity on the human PRLR (Fig. 7B).

Discussion

In two recent studies (20, 28) Walker and colleagues reported that S179D-hPRL 1) does not induce the proliferation of Nb2 cells, 2) dramatically antagonizes the growth-promoting activity of WT hPRL on these cells, and 3) activates STAT5 without (or minimally) activating JAK2. In this report we show that the same hPRL analog 1) acts as an agonist of the PRLR in the Nb2 as well as in various human receptor-mediated bioassays, 2) lacks any antagonistic activity toward WT hPRL in any of these bioassays, and 3) is a potent activator of JAK2, STAT5, and MAPK. The goal of this study was thus to explore why opposing conclusions were drawn for this hPRL analog despite the fact that it was produced using the same recombinant technology and characterized using the same bioassay (Nb2 cells). We hypothesized that these differences could result from the intrinsic properties of protein preparations, the experimental procedures used for the cell-based assays, and/or different biological tools, *e.g.* antibodies.

Upon refolding of inclusion bodies, about 50% of hPRL molecules assemble in high MM aggregates containing both covalent and noncovalent multimers. As PRL polymers have been long known to display reduced biological activity (for a review, see Ref. 41), we routinely eliminate these multimers from refolded hPRL through a single gel filtration step (25, 26, 31–33, 42). As illustrated by SDS-PAGE analysis, this step also eliminates most contaminants. In contrast, Walker *et al.* performed all of their studies using refolded, nonchromatographically purified S179D-hPRL. These researchers justified omitting the purification step by claiming that limiting the time of protein overexpression (IPTG) to 2 h, instead of 4 h as recommended in the initial protocol (32), reduces the amount of high MM contaminants pelleting within inclusion bodies (20), and their protein preparations did not contain oligomeric forms based on nonreducing SDS-PAGE analysis (data not shown in Ref. 20). We disagree with the latter argument, as noncovalent multimers cannot be detected by denaturing electrophoresis. Moreover, as we obtained indistinguishable chromatograms whether IPTG induction was

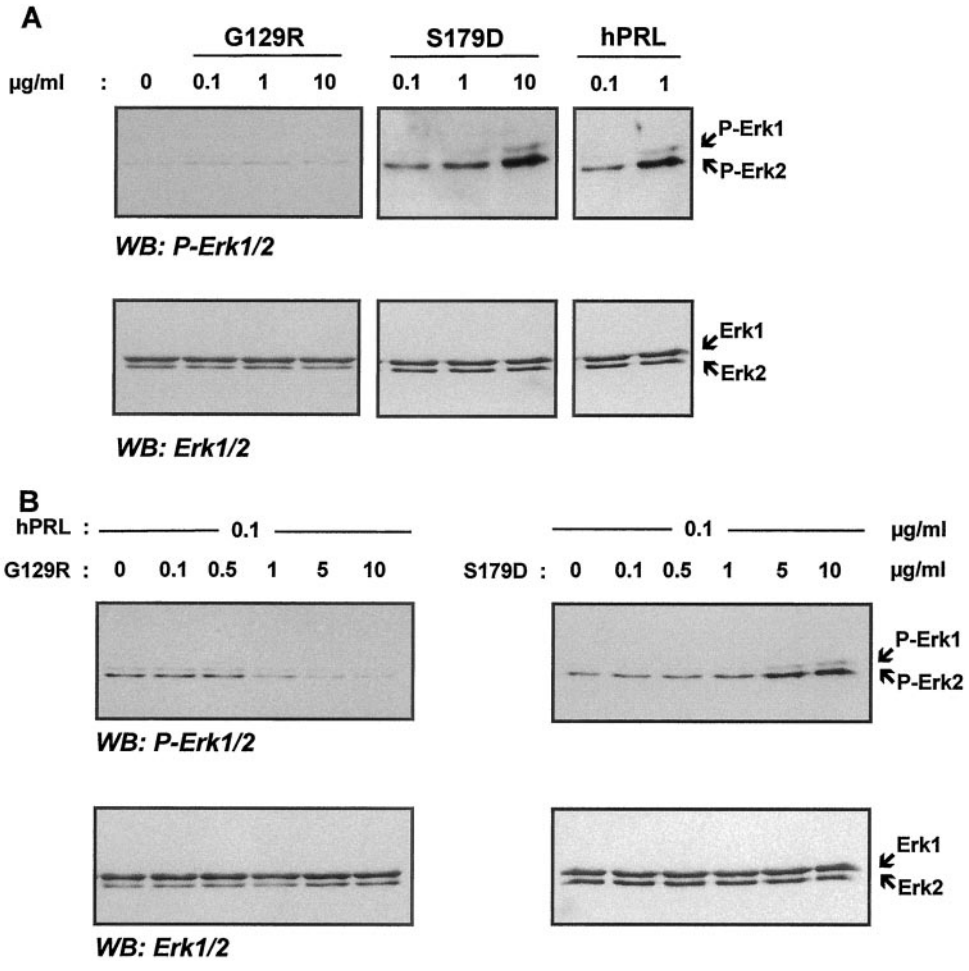

FIG. 7. Agonism and antagonism of hPRL and hPRL analogs on ERK1/2 in T-47D cells. T47D cells were cultured until semiconfluence in six-well plates, then stimulated (30 min) with various doses of WT hPRL or hPRL analogs as indicated (A, agonism). In antagonistic experiments (B), a fixed amount of WT hPRL (100 ng/ml) was competed with increasing doses of hPRL analogs. Fifty micrograms of total cell lysates were loaded per lane. Blots were then analyzed using anti-active MAP kinase antibodies (P-ERK1/2), then stripped and reprobed using anti-MAPK (ERK1/2) antibodies as indicated at the *bottom* of each panel. S179D-hPRL is able to induce tyrosine phosphorylation of MAPK to a level similar to, albeit slightly lower than, WT hPRL (compare 1 μg/ml concentration for both). In contrast, using identical time exposure for autoradiography, G129R-hPRL fails to activate MAPK as previously reported (27). In antagonism experiments, although G129R-hPRL inhibits the activation of MAPK induced by WT hPRL at a 10:1 ratio, S179D-hPRL has an additive effect on this pathway, reflecting its intrinsic agonistic activity in this assay.

performed for 2 h (not shown) or 4 h, we anticipate that the nonpurified hormone preparations used by Chen and colleagues are heterogeneous and contain a significantly amount of high MM proteins. Unfortunately, due to the absence of a gel filtration profile in their report, the true monomer/multimer ratio of their preparations cannot be assessed. Of more concern is that our nonpurified fraction probably contains bacterial contaminants normally eluted in the void volume of the molecular sieve, which may interfere with bioassays. The pronounced bell-shaped curves observed for nonpurified preparations compared with their purified counterparts may actually reflect such a PRL-independent effect resulting from bacterial contaminants presumably present in significant amount at high doses of hPRL analogs. Our policy is to always use purified proteins for characterizing hPRL analogs. Therefore, we will not go into detailed analysis of data obtained using our nonpurified material, because interpretation of such results could be misleading. Their use was solely to assess that the purification parameter alone did not support the contradiction between Walker's and our observations. Our data conclusively show that nonpurified S179D-hPRL acts as an agonist in the Nb2 proliferation assay. These observations were further confirmed, first using the luciferase bioassay developed in our laboratory and mediated by the homologous (human) PRLR, and second by monitoring T-47D cell proliferation or activation of the MAPK cascade known to be triggered by PRL in this human mammary tumor cell line. Depending on bioassay sensitivity, S179D-hPRL was clearly able to activate the hPRLR with an efficiency decreased by approximately 1 log unit, in perfect agreement with its 20-fold reduced binding affinity for this receptor. In summary, the discrepancy between both reports cannot result from different intrinsic properties of the protein preparations used.

For 20 yr, monitoring Nb2 cell proliferation has been established as the reference bioassay for quantifying lactogen activity (37, 43). Although the classical experimental procedures recommend using 10^5 cells/ml on the starting day of the assay, Walker and colleagues used 4 times less cell density in their bioassays, which we assumed may have influenced some characteristics of cell responses to hormone stimulation. Supporting this hypothesis, Chen *et al.* (20) reported unusual dose-response curves, with maximal response peaking at 200–400 pg/ml, whereas normally maximal cell growth is seen at 1–2 ng/ml hPRL (37). Still more confusing, they observed hPRL self-antagonism (reflected by bell-shaped curves) at concentrations as low as 1.6 ng/ml (20), whereas we (31) and many others (44–47) failed to detect any self-antagonism of lactogens on Nb2 cells at much more elevated concentrations (up to 1 μg/ml). To clarify this issue, we repeated all of our experiments following Chen's protocol (2.5×10^4 cells/ml), and our results appeared qualitatively

similar (not shown), *i.e.* that S179D-hPRL (purified or not) acted as a weak agonist on Nb2 cell proliferation.

As a first conclusion regarding agonistic assays, all of our data support that the agonistic properties of S179D-hPRL are real and were missed in Walker's reports. The most probable reason for this misinterpretation is that their bioassays were performed using an inappropriate concentration range of ligand. In fact, the growth-promoting effect of S179D-hPRL in the Nb2 assay occurs between 1–100 ng/ml, whereas the concentrations tested by Chen and colleagues did not exceed 1.6 ng/ml. This hypothesis was definitely confirmed when a S179D-hPRL preparation provided by Walker was shown in our hands to exhibit agonistic properties very similar to those of our preparation (not shown) in both Nb2 and HL5 assays.

As discussed previously (25), Nb2 cell proliferation is an extremely sensitive bioassay and therefore is probably not the most appropriate bioassay for highlighting the antagonistic properties of hPRL analogs. For example, although G129R-hPRL (25–27) and G120R-hGH (or G120K-hGH) (24, 39, 48) have been characterized as potent antagonists toward the PRLR in various cell-based assays, both are weak agonists and fail to antagonize WT lactogens in the Nb2 cell proliferation assay (31, 39, 44). This can be explained by the fact that maximal Nb2 cell proliferation is achieved at very low receptor occupancy (49), as highlighted by immunoblot analyses where maximal activation of signal transducers (*e.g.* JAK2 and STAT5) requires concentrations as high as 500 ng/ml, when 1 ng/ml hPRL is sufficient to promote maximal cell proliferation. Hence, PRL/GH analogs whose ability to dimerize the receptor is strongly, but not totally, abolished maintain the ability to induce receptor dimer formation to a minimal level, sufficient to promote Nb2 cell division (50). In view of this experimental background, the highly potent antagonistic properties of S179D-hPRL in the Nb2 assay reported in Chen's study appeared intriguing (20). They were still more questionable in view of the agonistic properties exhibited by S179D-hPRL in our hands. Expectedly, we failed to observe any evidence for antagonism when this analog was tested in competition experiments in the Nb2 assay. To circumvent the paradoxical response of Nb2 cells to hPRL analogs otherwise acting as potent antagonists, we have recently developed the PRLR-LHRE transcriptional assay that previously allowed unambiguous identification of PRL/GH mutants exhibiting antagonistic properties (25, 26, 39). As the transcriptional response, in contrast to cell division, is not (or minimally) affected by intracellular limiting factors, one can consider that the luciferase dose-response curve parallels that of receptor dimer formation (25, 50). In this bioassay, S179D-hPRL clearly lacks any antagonistic activity, whereas G129R-hPRL, the most potent hPRL antagonist currently available, does antagonize WT hPRL. These observations were further strengthened using human mammary T-47D cells. We (27) and others (48) have previously shown that PRL antagonists inhibit lactogen-induced proliferation of mammary tumor cells as well as the major PRLR-triggered signaling cascades, including JAK2; STAT1, -3, and -5; and MAPKs (27). In this study we monitored the inhibition of MAPK by hPRL analogs. Clearly, whereas G129R-hPRL leads to extinction of the phosphorylation signal induced by the intermediate dose of hPRL, S179D-hPRL still increases MAPK activation. Therefore, we must conclude that S179D-hPRL is completely devoid of any detectable antagonistic activity. To understand why contradictory results were obtained, we repeated the experiments involving Nb2 cells using the protocol described in Chen's study (20). In our hands, using a cell density as low as 5×10^3 cells/ml appeared unreliable, as the OD_{450} values were so low that the variation in absorbance (change in OD_{450}, <0.1 U) was not significant. We repeatedly observed a small decrease in Nb2 cell response in a very narrow range of low concentrations using nonpurified hPRLs (including WT hPRL) in competition experiments. Although we cannot propose any molecular mechanism to support this observation, such a phenomenon limited in amplitude and in concentration range may be reminiscent of what Walker and colleagues interpreted as reflecting S179D-hPRL antagonism.

In a second report (28) the same group suggested that S179D-hPRL may activate alternative intracellular signaling cascades, although such pathways were not identified. In a more general fashion their data argued for a dissociation between JAK2 and STAT5 activation, in total contradiction with the conventional model of JAK-induced phosphorylation of STATs (51). Again, we absolutely failed to confirm these observations, as S179D-hPRL, purified or not, appeared able to induce JAK2 phosphorylation in a fashion similar to WT hPRL in Nb2 cells. A tentative explanation for the discrepancy between the results of Walker's group and ours might be that the level of JAK2 phosphorylation was, in general, low in their hands, but detectable (Fig. 3A in Ref. 28), which may have led the researchers to suggest the involvement of another kinase in STAT5 activation. Accordingly, we obtained stronger signals using the anti-JAK2 antibody from Upstate Biotechnology, Inc., compared with that from Santa Cruz Biotechnology, Inc., used in their study (data not shown), which, however, does not support the discrepancy, as the antibody from Santa Cruz Biotechnology, Inc., successfully detected JAK2 phosphorylation induced by WT hPRL (28). Moreover, we observed that G129R-hPRL induced only a limited level of JAK2 phosphorylation, demonstrating that our experimental procedures were sensitive enough to identify analogs exhibiting lower ability to activate this signaling cascade. According to these observations regarding agonistic effect, we failed to confirm the antagonistic activity of S179D-hPRL on hPRL-induced JAK2 activation previously reported by these researchers, whereas the antagonism of G129R-hPRL could be detected, in agreement with its inhibitory effect on PRLR signaling pathways recently demonstrated in breast cancer cells (this study and Ref. 27). The only difference repeatedly observed between S179D-hPRL and WT hPRL is a decrease in the pool of phosphorylated JAK2 coprecipitating with STAT5, which may reflect a difference in the stoichiometry and/or conformation of PRLR/JAK2/STAT5 complexes depending on the ligand (see below). Obviously, the importance of this observation will require further investigation, which, however, is out of the scope of the present study.

All of the results presented in this study clearly establish S179D-hPRL as an agonist (although weaker than hPRL), not as an antagonist, of the PRL receptor. However, our report raises many questions, the first of which is how this hPRL

analog should be categorized. Several arguments suggest that S179D-hPRL is a binding site 1 rather than a binding site 2 analog. Based on the three-dimensional model that we established (52), serine 179 is predicted to belong to helix 4, the region of binding site 1 containing the highest number of binding determinants (6). Moreover, the dose-response curves obtained for S179D-hPRL in the various bioassays investigated here are displaced to the right compared with WT hPRL and achieve a maximal response (or supramaximal, see below), two features that fit with alteration of binding site 1 (6, 33, 42, 50). Although S179D-hPRL can be considered as a site 1 analog, this does not necessarily mean that serine 179 is intrinsically involved in an interaction with the PRLR. One of the most powerful methods for identifying binding determinants of PRLs or GHs has proved to be alanine scanning (for review, see Ref. 6). Although Chen *et al.* (20) reported that alanine replacement of serine 179 abolishes hPRL activity, which would argue that this residue is functionally relevant, we cannot exclude that the putative agonistic activity of the S179A-hPRL analog could have been missed, as it was for S179D-hPRL (see above). In contrast to all binding determinants identified to date (6), serine 179 is predicted to point toward the hydrophobic core of the protein (52), which presumably precludes this residue from directly interacting with the receptor. This structural hypothesis is corroborated by isoelectric focusing experiments showing that the pI of S179D-hPRL is identical to that of WT hPRL despite the addition of a negative charge (Asp for Ser). We expect that Asp^{179} (and Ser in WT hPRL) is buried and that its putative charge does not influence electrophoretic mobility. If true, this would suggest that the reduced agonism of S179D-hPRL would not result from the mutation of Ser^{179} *per se*, but, rather, from the effect this mutation has on overall protein structure. The estimation of S179D-hPRL apparent MM using analytical gel filtration did not reveal any detectable change with respect to WT hPRL, but this approach is obviously not very sensitive to small structural variations. Circular dichroism previously enabled us to detect structural disturbance of hPRL analogs (*e.g.* C58A or S26W) (31, 33). Using this approach, we failed to detect any significant alteration of secondary structure content of S179D-hPRL, as its spectrum and calculated helicity were not significantly different from those of WT hPRL (this study and Refs. 31 and 33). In agreement with Chen and colleagues, who used RIA recognition to monitor conformational disturbances, we thus conclude that the S179D substitution does not dramatically influence the overall hPRL structure.

As Ser^{179} is presumably buried and, in addition, mutation of this residue leads to reduction of affinity and of agonistic activity without any detectable structural alteration, we hypothesize that aspartate substitution results in local disturbance of regions involved in binding site 1, most probably of helix 4. Recent investigation of Walker's group indicates that Ser^{179} can be phosphorylated *in vitro* in the human hormone (Lorenson, M. Y., and A. Walker, personal communication), as already demonstrated for the homologous Ser^{177} in rPRL (18), which, however, does not mean that these residues actually are phosphorylated in the pituitary *in vivo*. Elucidating the mechanism by which an amino acid predicted to be buried inside the protein becomes accessible to a kinase remains open to investigation. Whether S179D-hPRL actually exhibits the same properties as a putative S179-phosphorylated hPRL, thereby justifying being referred to as a molecular mimic of phosphorylated hPRL remains an open question (20, 53).

The final and probably most important questions relate to the intrinsic properties that S179D-hPRL exerts *in vivo* when injected to animals. Within the last year, several reports on the effect of S179D-hPRL in various rodent models have been presented. Nonexhaustively, S179D-hPRL was reported to reduce the tumor incidence of cancer prostate cells injected into nude mice (54) and in rats to alter maternal behavior in nulliparous female (55) and to affect T cell survival (56), which is in good agreement with the antagonistic properties claimed by Walker and colleagues from their *in vitro* studies (20, 28), but obviously does not agree with our conclusions. In contrast, S179D-hPRL was also shown to promote lobuloalveolar differentiation and casein expression during rat pregnancy (57). Moreover, a very recent publication by the same group described S179D-hPRL as an even more potent agonist than WT hPRL on the inhibition of osteoblastic alkaline phosphatase activity and bone development in rats (58), which fits with the agonistic activity demonstrated for this analog in the present work. Although one of our concerns about the relevance of these preliminary *in vivo* studies is whether nonpurified proteins obtained from bacteria are a suitable material to be injected into animals, especially for investigations of immunological parameters, the paradoxical properties of S179D-hPRL both *in vitro* and *in vivo* obviously argue that this hPRL mutant should be viewed with much caution and certainly not as a full antagonist as suggested previously (20, 55, 56). Unexpectedly, our transcriptional bioassay revealed that although S179D-hPRL behaves as a slightly weaker agonist than hPRL (dose-response curve displaced to the right), it induces a luciferase response of higher amplitude (~125%) compared with WT hPRL, thereby displaying superagonistic properties *in vitro*. As this phenomenon was also observed using the hormone preparation provided by Walker (not shown), it may correlate with the superagonistic activity reported for S179D-hPRL on the inhibition of bone formation (58), although this reported effect would be opposed to the reduced bone development we have reported in PRL receptor knockout mice (59). This suggests that once the functional hormone-receptor complex is formed (which requires higher hormone concentration due to reduced affinity), S179D-hPRL might be able to trigger signaling cascades more efficiently than the natural ligand. Whether this property is related to prolonged signal transduction, a different k_{on}/k_{off} affinity constant, conformational changes in the PRLR, or some other molecular feature remains to be explored.

In conclusion, the present report clearly establishes S179D-hPRL as a PRLR agonist and suggests that the antagonistic properties claimed for this analog were deduced from inappropriate bioassays. Although it is assumed that phosphorylated PRL plays a role in autoregulation of PRL secretion from the pituitary or is an intracellular component acting as a regulator of trafficking and/or storage of PRL molecules, the physiological relevance of this PRL variant remains poorly understood (12). Based on injection of S179D-hPRL to

pregnant rats, it was recently proposed that the ratio of nonphosphorylated *vs.* phosphorylated hPRL, rather the latter *per se*, may be physiologically relevant. We strongly feel that extrapolation of findings with S179D-hPRL to the potential physiological relevance of phosphorylated hPRL *in vivo* remains to be explored and should await the definite demonstration that the S179D mutation actually confers properties similar to those specifically exhibited by phosphorylated PRL.

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