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Physico-chemical and biological characterizations of two human prolactin analogs exhibiting controversial bioactivity, synthesized in Chinese hamster ovary (CHO) cells

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Abstract

The synthesis, purification and characterization of G129R–hPRL and S179D–hPRL, the two better-studied antagonists of human prolactin (hPRL), is described. Both of these have been expressed for the first time, in their authentic form, by a stable CHO cell line, at secretion levels of 7.7 and 4.3 μg/10^6 cells/day, respectively. Previous studies had shown that these hPRL analogs, when produced in bacterial cytoplasm, consistently contained misfolded forms and multimers according to the specific denaturation, refolding and purification conditions. These versions also have an N-terminal extra methionine. An extensive physico-chemical characterization was carried out after a practical two-step purification process and included SDS–PAGE and Western blotting analysis, matrix-assisted laser-desorption ionization time-of-flight mass spectral (MALDI–TOF–MS) analysis, high-performance size-exclusion chromatography (HPSEC) and reversed-phase high-performance liquid chromatography (RP–HPLC). This last technique revealed a considerable difference in hydrophobicity due to a single amino acid substitution, with S179D–hPRL less ($t_{RR} = 0.85 \pm 0.010$) and G129R–hPRL more ($t_{RR} = 1.10 \pm 0.013$) hydrophobic than hPRL, where t_{RR} is the relative retention time. The biological characterization was based on further refinement of a sensitive proliferation assay using the pro-B murine cell line (Ba/F3) transfected with the long form hPRL receptor cDNA such that the minimal detectable dose was 0.04 ng of hPRL/mL, the Ba/F3-LLP assay. On the basis of this assay, the relative residual agonistic activity of these two products, determined against a hPRL international standard in four independent assays, was 53×10^{-3} for S179D–hPRL and 70×10^{-5} for G129R–hPRL. We believe that the present synthesis and characterization could be extremely helpful for studies of these two proteins, which have been reported to antagonize tumor growth-promoting effects of hPRL in vivo in animal models of breast and prostate cancer.
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Keywords: Prolactin; Antagonist; G129R–hPRL; S179D–hPRL; CHO cells; Ba/F3 assay; Breast cancer; Prostate cancer

Human prolactin (hPRL), a hormone primarily secreted by the anterior pituitary gland has a wide spectrum of biological functions [1–3]. This hormone, however, has also been shown to be expressed by many extra-pituitary sites, including mammary epithelial cells, and is able to stimulate cell proliferation via an autocrine–paracrine loop [4–7]. Considering that the PRL receptor (PRLR) is expressed in practically all mammary epithelial cells, that its expression level is in general higher in tumors [8–10] and that PRL induces the expression of various proteins that are involved in breast cancer progression such as cyclin D1 [11] or IGF-2 [12], the

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development of specific PRLR antagonists is obviously extremely important, especially for blocking the proliferative effects of extra-pituitary hPRL[1] production [13,14].

Up to now, three fundamental hPRL antagonists have been described in the literature. The hGH analog G120K-hGH is a potent antagonist of both the hGHR [15] and the hPRLR [16], therefore its potential clinical use may be limited because it does not antagonize a specific receptor. Also, it requires substantial concentrations of zinc to bind tightly to the PRLR, a situation that may modulate its efficiency in various tissues in vivo. G129R–hPRL, the first PRL-based antagonist of the hPRLR, was developed considering that Gly 129 of hPRL is invariant among PRLs and corresponds to Gly 120 of hGH or Gly 119 of bGH [17–20]. Several antagonists based on the G129R mutation, have been engineered more recently [21–23]. The second type of antagonist, is S179D–hPRL, a molecular mimic of phosphorylated PRL, produced by substituting an aspartate residue for the normally phosphorylated Ser 179 [24,25].

Although studies of their in vitro activity have given rise to some controversy concerning their agonistic and/or antagonistic properties [13,14,20,25–30], both G129R–hPRL and S179D–hPRL have demonstrated anti-tumor effects in vivo [31,32] and have the potential to contribute to the treatment of human breast and prostate cancer in the near future.

The two hPRL analogs G129R–hPRL and S179D–hPRL have been obtained in *Escherichia coli* cytoplasm in two different laboratories [18,25] and submitted to inclusion body extraction and purification, to denaturation and refolding of the protein and to purification of the refolded protein. The efficiency of the refolding process is not easily controllable and the presence of incorrectly folded forms and disulfide-linked multimers of the mutants makes it difficult to attain a consistent product, a consistency required by regulatory agencies for human therapeutics. Besides the time-consuming procedure and the presence of an undesired N-terminal methionine, contamination by endotoxin has also been considered as a potential problem in this type of product [26,33]. Endotoxins, which are the major component of the outer membrane of Gram-negative bacteria, can have potent effects on cell proliferation and apoptosis [34], with possible interference with agonistic or antagonistic bioassays. For these reasons, we decided to express the two hPRL analogs in Chinese hamster ovary (CHO) cells, utilizing a mammalian cell expression system already used in previous work for hPRL synthesis [35] and always checking for possible endotoxin contaminations. After a practical two-step purification process, these production procedures allowed us to perform extensive physico-chemical characterization of the two hPRL analogs. Our ultimate goal was to determine and reliably quantify their biological activity to compare their intrinsic functional characteristics and better evaluate their potential clinical applications. Based on the extensive studies carried out by Kinet et al. [27] and by Bernichtein et al. [13] on the new and existing bioassays for human lactogens, we decided to set up an extremely sensitive assay aimed at determining the agonistic potency of these two analogs towards their homologous receptor. This assay was based on the proliferation of a pro-B murine cell line (Ba/F3), stably expressing the long form of the human PRL receptor (hPRLR) and grown in the presence of a very low hPRL concentration: the Ba/F3 LLP assay.

Materials and methods

Construction of expression vectors

The expression vectors for G129R–hPRL and S179D–hPRL were derived from p658-hPRL [35]. The mutated DNA sequences were obtained by PCR, the correct mutation being confirmed by DNA sequencing by the method of Sanger et al. [36]. After digestion with endonucleases *Eco*RI and *Bam*HI, the mutated fragment was introduced in place of a fragment corresponding to part of the sequence of the authentic hPRL, the resulting vectors being named p658–G129R–hPRL and p658–S179D–hPRL.

The synthetic primers used were:

(1) 21 mer, sense for G129R–hPRL construction

5′CTTCTAGAGCGCATGGAGCTG3′

(2) 39 mer, antisense for G129R–hPRL construction, also introducing *Bam*HI restriction site and the stop codon

5′GATCGATCGGATCCTTATCAGCAGTTGTT GTTGTGGATG3′

(3) 92 mer, antisense for S179D–hPRL construction also introducing *Bam*HI restriction site and the stop codon

5′GATCGATCGGATCCTTATCAGCAGTTGTTGT TGTTGATGATTCGGCACTTCAGGAGCTTGAGA TAATTGTCGATTTTATGGTCATCCCTGCG3′

(4) 99 MER, sense, introducing *Nde*I restriction site and hPRL signal peptide in the final construction for the two analogs.

5′GATCGATCGATCCATATGAAAAGATCCTGG CGTTAGCTGCGCTGACTACCGTTGTATTCTCTG CGTCCGCCTTCGCTTTGCCCATCTGTCCCGGCG GG3′

To obtain the sequence of G129R–hPRL, the GGC codon, corresponding to amino acid 129 (glycine) was

[1] *Abbreviations used:* hPRL, human prolactin; hPRLR, human PRL receptor; CHO, Chinese hamster ovary; MEM, minimal essential medium; FBS, fetal bovine serum; SA, sinapinic acid; MTS, [3(4,5-dimethylthiazol-2-yl)-5(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolin; PBS, phosphate-buffered saline; MALDI–TOF–MS, matrix-assisted laser-desorption ionization time-of-flight mass spectrometry; RP–HPLC, reversed-phase high-performance liquid chromatography; HPSEC, high-performance size-exclusion chromatography.

changed into CGC (arginine). Two PCRs were utilized for this analog. Reaction 1: synthesis of the primer antisense utilized for reaction 2 employing primers 1 and 2. Reaction 2: synthesis of the complete sequence, employing the product of reaction 1 and primer 4.

To obtain the sequence of S179D–hPRL, the TCA codon, corresponding to amino acid 179 (serine) was changed into GAC (aspartate). The PCR utilized the primers 3 and 4.

Vector transfection and expression

CHO dhfr− cells (clone DUKX-B11) have been described by Urlaub and Chasin [37]. Cells were maintained in minimal essential medium (MEM) supplemented with 20 μg/mL gentamycin and 5% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA). Transfection was carried out by the calcium phosphate precipitation technique [38] and, 2 days post-transfection, cells were seeded into 100 mm culture dishes at 5×10^5 cells/dish, using α-MEM with L-glutamine and without ribonucleosides and deoxyribonucleosides, supplemented with 10% dialysed FBS as selective medium. After 2 weeks, the selected transformed colonies were isolated using cloning cylinders and, following several subculture steps directed to culture amplification, the clones were analysed for expression using a radioimmunoassay for hPRL. The whole selection and amplification process took approximately 45 days. The most productive clone obtained for each analog was used for the laboratory production.

Radioimmunoassay

The amount of secreted hPRL and analogs was determined by a double-antibody liquid-phase radioimmunoassay using reagents prepared at IPEN-CNEN (São Paulo, Brazil) as described previously [39]. NIDDK-hPRL-RP-I and NIDDK-anti-hPRL-3 were provided by Dr. A.F. Parlow from the National Hormone and pituitary program (Torrance, CA, USA). Human-blood-based immunoassay quality controls (Dade® Tri-level, Baxter Diagnostic, Deerfield, IL, USA) were used for each standard curve.

Analogs purification

To generate conditioned medium for the purification of the two analogs, CHO transfected cells were grown on 100 mm culture dishes in CD–CHO serum free medium (Invitrogen, Carlsbad, CA, USA), containing 20 μg/mL of gentamycin and supplemented with 10% (v/v) dialysed FBS (Invitrogen, Carlsbad, CA, USA), until 40–50% confluence was reached. The culture medium was then changed into the same medium from which serum had been omitted and the conditioned medium was harvested every 24 h, for 10 days and stored at −80 °C.

The analog present in conditioned culture medium was purified using a modified version of the method described by Soares et al. [35]. A two-step purification process was used: SP–Sepharose fast flow followed by a size exclusion chromatography employing HPSEC as preparative column. Briefly, conditioned medium was adjusted to pH 5.0 using acetic acid. The material was then applied onto the SP–Sepharose fast flow column (Pharmacia, São Paulo, Brazil) equilibrated in 50 mM sodium acetate (pH 5.0), UV absorbance was monitored at 280 nm. After washing with the same buffer, the column was washed with 50 mM sodium acetate (pH 5.0), 90 mM NaCl. The protein of interest was eluted from the column with 25 mM Hepes (pH 8.0). Different fractions containing the analog were analysed by HPSEC and the most concentrated fractions purified on the same HPSEC column, working this time as a preparative column. The maximum volume applied for each HPSEC preparative run was 500 μL. A final pool, derived from HPSEC purification, was quantified for bioassay purposes via HPSEC and RP–HPLC and stored at −80 °C.

Gel electrophoresis and Western blot analysis

Discontinuous SDS–PAGE, based on 15% polyacrylamide gels, was carried out under non-reducing conditions as described [40]. Coomassie brilliant blue G-250 (USB, Cleveland, OH) was used for staining and the molecular mass markers were from Amersham pharmacia biotech (Piscataway, NJ, USA). Western blot analysis was performed using ^{125}I-labelled protein A [34].

Reversed-phase high-performance liquid chromatography (RP–HPLC)

A Shimadzu Model SCL-10A HPLC apparatus coupled to a SPD-10AV UV detector (Shimadzu, MD, USA) was used, employing the Class VP software, also from Shimadzu. The column was a C4 Vydac 214TP54 (25 cm × 4.6 mm ID, pore diameter of 300 Å and particle diameter of 5 μm) with a guard column (Vydac 214FSK54) between the sample injector and the main column and a silica precolumn packed with LiChrosorb Si-60, 7.9–12.4 μm (Merck, Darmstadt, Germany) located between the pump and the injector. All Vydac columns were purchased from Grace Vydac (Hesperia, CA, USA). The mobile phase consisted of 71% Tris–HCl buffer (50 mM, pH 7.5) and 29% *n*-propanol, as described by Dalmora et al. [41], with a flow-rate of 0.5 mL/min, detector wavelength at 220 nm, column temperature maintained at 45 °C and a sample volume of 25–200 μL [42]. A relative retention time (t_{RR}), calculated on the basis of the t_R of hPRL obtained on the same day, where $t_{RR}x = t_R x / t_R$ hPRL for a given form *x*, was used for identifying and comparing the two analogs.

High-performance size-exclusion chromatography (HPSEC)

HPSEC was carried out with the same Shimadzu apparatus, processing 5 to 500 μL of sample on a TosoHaas

(Montgomeryville, PA, USA) G2000 SW column (60 cm × 7.5 mm ID, particle size of 10 μm and pore size of 125 Å) coupled to a 7.5 cm × 7.5 mm ID SW guard column. The mobile phase was 0.025 M ammonium bicarbonate, pH7.0, with a flow-rate of 1.0 mL/min [41]. This methodology was used as an analytical and preparative technique.

Mass spectrometry

MALDI–TOF–MS analyses of hPRL, G129R–hPRL and S179D–hPRL were carried out using sinapinic acid (SA) as the matrix and a Voyager-DE BioSpectrometry Work-station from Applied Biosystems (Foster City, CA, USA).

In vitro bioassay

Ba/F3 cells are murine pro-B cells, dependent on IL-3 to proliferate. As described by Bernichtein et al. [13], these cells were transfected with a plasmid containing the sequence encoding the human PRL receptor (hPRLR) long isoform and a gene for geneticin resistance. The substitution of hPRL for IL-3 in the routine culture medium permitted the selection of hPRL-dependent cells. A stable population with the maximum proliferation at 10 ng/mL of hPRL (BaF3-LP) (low PRL) was obtained earlier [13]. After a similar treatment with a lower PRL concentration (1 ng/mL), a more sensitive population was selected, namely Ba/F3-LLP (low-low PRL), whose maximum proliferation was in the presence of 1 ng/mL of PRL in the culture medium. A homologous proliferation bioassay, employing Ba/F3-LLP cells, was thus developed to determine the activity of each analog in comparison with hPRL.

Ba/F3-LLP cells were routinely maintained in suspension in RPMI-1640 medium supplemented with 10% heat-inactivated FCS; 2 mM glutamine; 50 U/mL of penicillin; 50 μg/mL of streptomycin; 700 μg/mL of geneticin and 1 ng/mL of recombinant hPRL (rhPRL). Before carrying out the proliferation assay, cells were starved for 6-h in RPMI-1640 medium containing 1% heat-inactivated FCS, 2 mM glutamine; 50 U/mL of penicillin; 50 μg/mL of streptomycin; 700 μg/mL of geneticin, and no rhPRL. Cells were then distributed in flat bottom 96 well-plates at a density of 5×10^4 cells/well in a final volume of 200 μL. After 72 h at 37 °C and 5% CO_2, the presence of viable cells was assessed using the MTS assay [25]. Briefly, 2 mg/mL MTS dye [3(4,5-dimethylthiazol-2-yl)-5(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolin (Promega Corp., Madison, WI, USA)] in PBS (phosphate-buffered saline) was mixed in a 20:1 ratio (vol/vol) with phenazine methosulfate (Sigma, St. Louis, MO, USA), 0.92 mg/mL in PBS. Twenty microliters of the mixture were then added to each well, and 2-h after incubation at 37 °C the absorbance at 490 nm was read in a microplate reader (Dynatech, Model MR4000, Chantilly, VA, USA). Human recombinant PRL, Chemical Reference Standard (CRS-WHO) (2.2 mg rhPRL/mL) was used as reference preparation. The bacterially (cytoplasmic)-derived analogs G129R–hPRL and S179D–hPRL, synthesized as previously described [18,25], were obtained from Dr. Vincent Goffin and Dr. Ameae M. Walker laboratories.

Results

The vectors p658-hPRL, p658–G129R–hPRL and p658–S179D–hPRL were used for transfecting CHO dhfr− (clone DUKX-B11) as previously described. The best clones obtained provided the expression levels presented in Table 1. Interestingly, while both G129R–hPRL and WT-hPRL, were also successfully expressed in *E. coli* periplasm (expression yield for G129R–hPRL 0.8 μg/mL/A_{600}), attempts to produce S179D–hPRL this way in bacteria at useful yields repeatedly failed in our hands. Before starting laboratory production of the proteins of interest, a study was carried out to determine the most effective culture medium, considering not only secretion efficiency, but also the presence of contaminant proteins. Fig. 1 shows the result of this analysis for different conditioned media, carried out via SDS–PAGE and Western blot analysis. While secretion efficiency was approximately of the same level for CHO–S–SFM–II, with or without nucleosides, and for CD–CHO medium, the electrophoretic purity of hPRL obtained with the latter was clearly higher. This led us to choose the CD–CHO medium for all our production of hPRL and its two analogs.

To be able to characterize the recombinant products, a fast and practical two-step purification process was set up and applied to hPRL and its analogs. This was based on an initial chromatographic purification on a cationic exchanger (SP–Sepharose fast flow), followed by a rapid HPSEC on the same Tosohaas G2000 SW long column (60 cm × 7.5 mm ID), that we also use for routine analytical purposes. Fig. 2 shows a typical SDS–PAGE analysis of the fractions eluted from SP-Sepharose Fast Flow and containing, in this case, G129R–hPRL. Considering band intensity and purity, we can already consider fraction #29 to be the one to be selected. This was further confirmed by RP–HPLC and HPSEC analyses (Fig. 3). This fraction was then recovered for the next purification step (Fig. 4A). These two simple purification steps perfectly satisfied the objective to rapidly obtain a highly purified product (>97.3%), that could be readily characterized from physico-chemical and biological points of view (Fig. 4B). The electrophoretical

Table 1
Expression levels of the best clones of CHO obtained with p658-hPRL, p658–G129R–hPRL and p658–S179D–hPRL, determined by radioimmunoassay

Preparation	Expression level ± SD[a] (μg/10^6 cells/day)	RSD[b] (%)
hPRL	13.5 ± 1.89	14.0
G129R–hPRL	7.7 ± 0.71	9.2
S179D–hPRL	4.3 ± 0.60	13.9

[a] Median ± standard deviation, each value being the average of $n = 2$ determinations.

[b] Relative standard deviation, expressed as percentage of the mean.

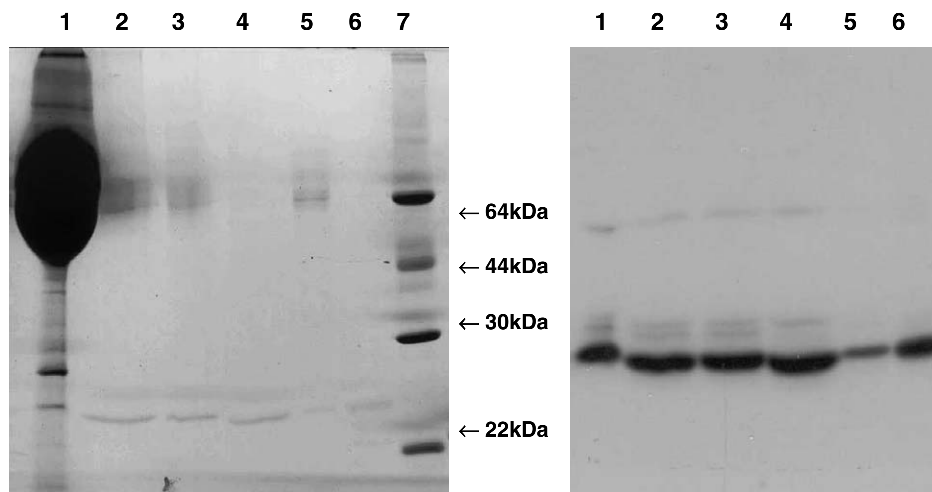

Fig. 1. SDS–PAGE (A) and Western blot (B) analysis under non-reducing conditions of different media employed for culturing hPRL-secreting CHO cells. Lane 1, α-MEM with 10% dialyzed bovine fetal serum. Lane 2, CHO–S–SFM–II without nucleosides. Lane 3, CHO–S–SFM–II with nucleosides. Lane 4, CD–CHO medium. Lane 5, α-MEM without fetal serum. Lane 6, pituitary hPRL reference preparation (NIDDK), 5 μg. Lane 7, molecular mass markers.

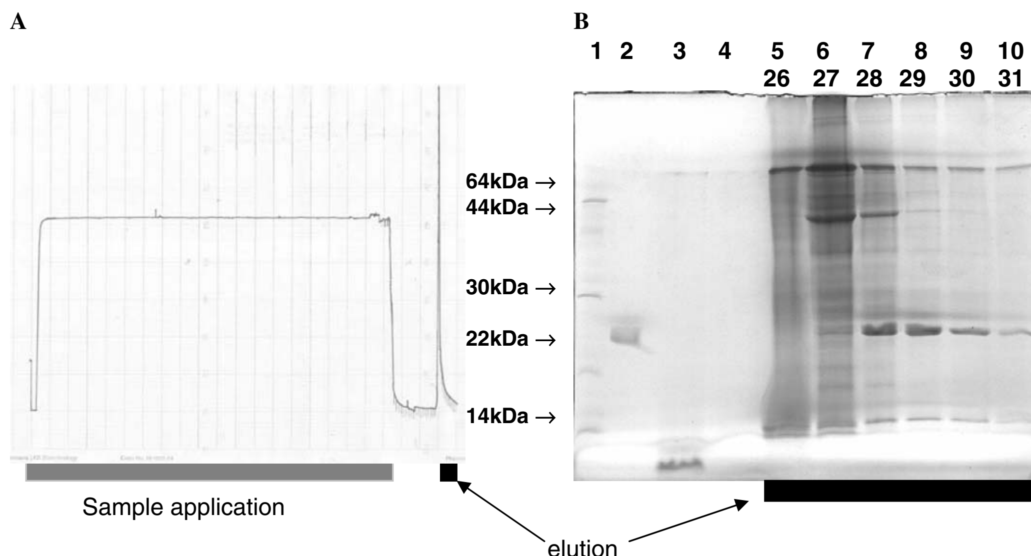

Fig. 2. Example of chromatographic purification on SP–Sepharose fast flow of the analog G129R–hPRL from CHO culture medium (A) and SDS–PAGE analysis, under non-reducing conditions, of the eluted fractions (B). Lane 1, molecular mass marker. Lane 2, *E. coli*-secreted hPRL. Line 3, applied sample. Lane 4, non-retained fraction. Lanes 5–10, eluted fractions #26–31.

and immunological purity of the three final products (hPRL, G129R–hPRL and S179D–hPRL) was then confirmed by the SDS–PAGE and Western blot analysis shown in Fig. 5, which also makes a comparison with the two proteins (hPRL and G129R–hPRL) obtained in *E. coli* periplasm. The relative retention times (t_{RR}) obtained for the two analogs by HPSEC and RP–HPLC against the retention time of hPRL obtained in *E. coli*, are presented in Table 2. Samples of hPRL obtained in CHO and of G129R–hPRL obtained in *E. coli* were also included in the study. We can observe that, while on HPSEC there is no significant difference between the different preparations, on RP–HPLC there is a highly significant difference ($P < 0.001$) between the t_{RR} of G129R–hPRL (+11.1%) and of S179D–hPRL (−14.1%) and those of hPRL (CHO), indicating different levels of hydrophobicity between these molecules.

Having obtained quite accurate molecular mass determinations for recombinant human thyrotropin and its subunits in previous work via MALDI–TOF–MS analysis [44], we also applied this sensitive methodology to the present products. Having introduced the correct mutations by PCR into the hPRL coding sequence to obtain the secreted G129R–hPRL and S179D–hPRL, we confirmed that this type of spectral analysis is an extremely useful identity test even for the case of our analogs. In Table 3 the relative molecular masses obtained via MALDI–TOF–MS analysis are compared to the calculated mean values of the three proteins of interest and, as we can observe, the differences

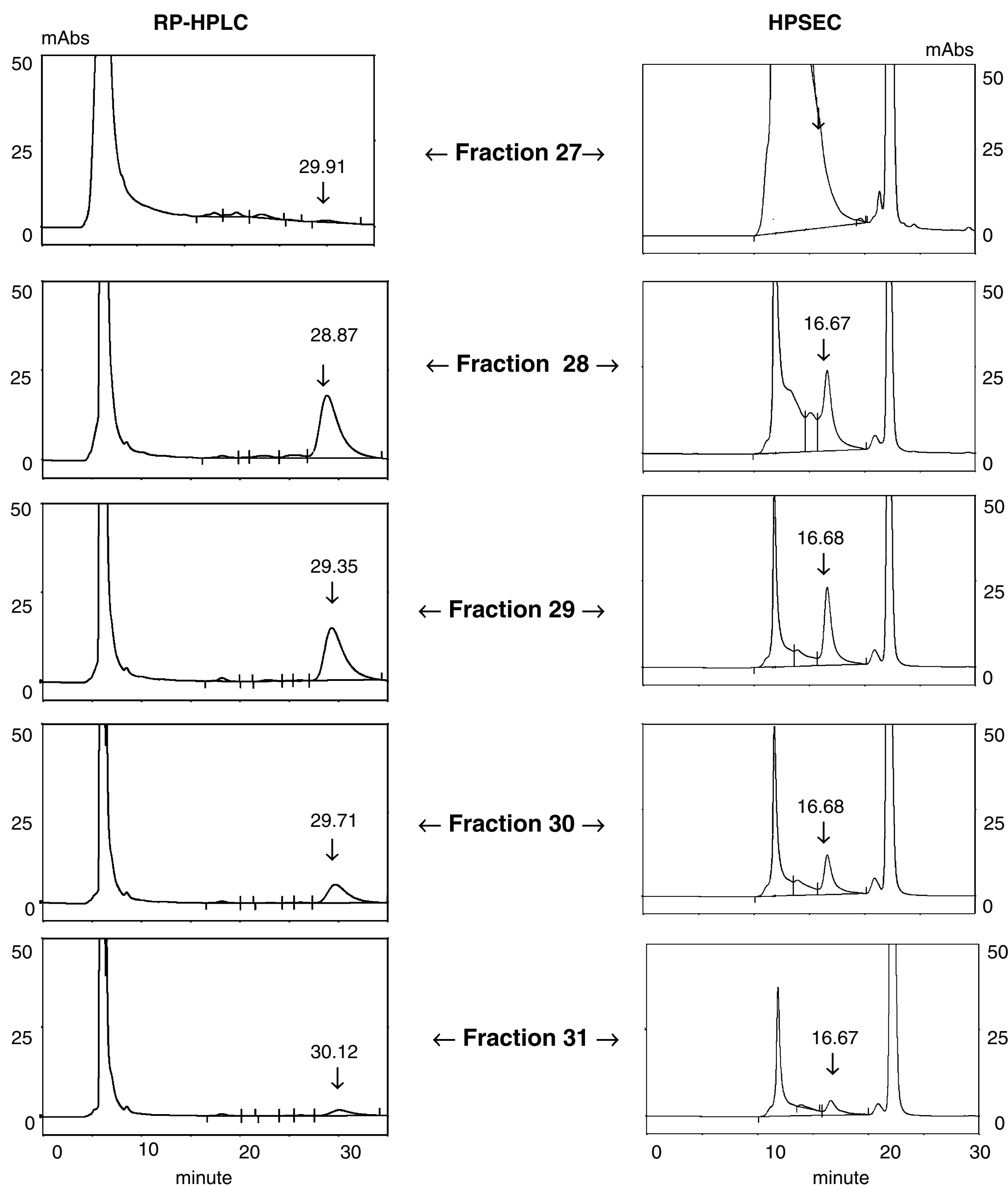

Fig. 3. RP–HPLC and HPSEC analysis of the fractions eluted from SP–Sepharose fast flow. The arrows indicate the peak of G129R–hPRL.

between the theoretical and experimental values are, in general, <0.1% while the expected difference between hPRL and the two analogs, 0.4% and 0.12%, respectively, for G129R–hPRL and S179D–hPRL is confirmed. An example of the spectra obtained for each protein analyzed is also reported in Fig. 6.

Finally, hPRL and the two analogs were also characterized and quantified via the highly sensitive and homologous bioassay, based on the proliferation of Ba/F3-LLP cells (Fig. 7). The minimal detectable dose, calculated according to Rodbard's formulation [43], was 0.036 ± 0.024 ng/mL ($n = 5$), i.e. approximately 15-fold lower than that obtained by us in the Ba/F3-LP assay: 0.54 ± 0.23 ng/mL ($n = 4$). As expected, WT hPRL displayed a bell-shaped curve, with maximal activity at 0.5 ng/mL. The dose–response curve of S179D–hPRL was displaced towards the high concentrations by slightly less than two log units, and was also able to stimulate cell proliferation to the maximal level. The dose–response curve of G129R–hPRL was even more displaced to the high concentrations and also reached maximal proliferation. The statistical parameters obtained for these three ligands are presented in Table 4. Whether the slopes of the dose–response curve or the respective ED_{50} are considered, our data assess the reproducibility of the assay. A comparison between the proliferative activity of the bacterially (cytoplasmic)- and CHO-derived analogs in Ba/F3-LLP assay was carried out (Table 5). No significant difference was observed for the two analogs (S179D–hPRL

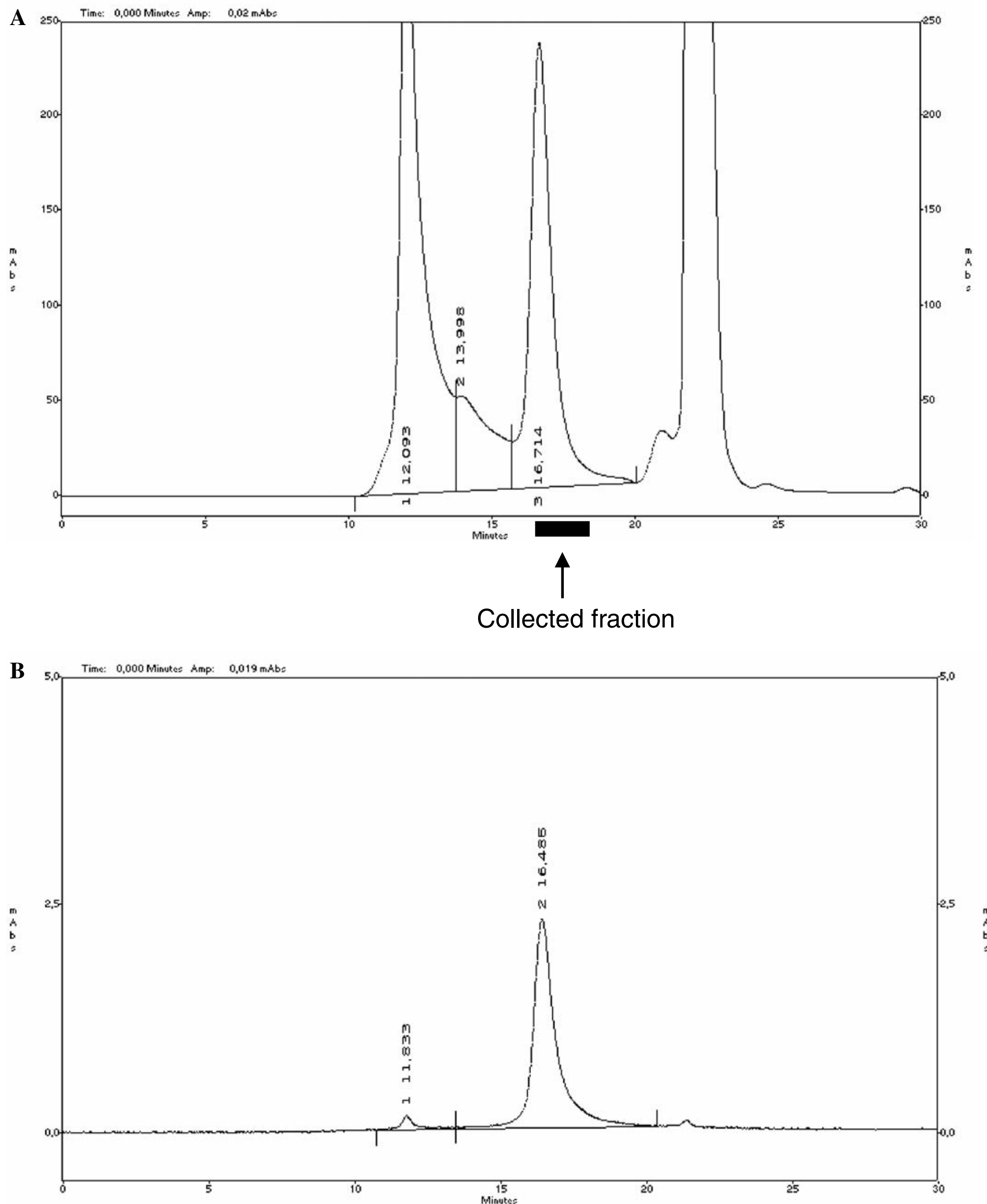

Fig. 4. Example of HPSEC purification of G129R–hPRL obtained in fraction #29 (see Figs. 2 and 3). (A) Five hundred microliters of fraction #29, obtained from SP–Sepharose fast flow and applied to a Tosohaas G2000 SW column (60 cm × 7.5 mm ID). (B) Fifty microliters of the fraction collected in (A) analysed on the same column.

and G129R–hPRL) when obtained either in bacterial cytoplasm or in their authentic form from CHO cell conditioned medium.

Discussion

For the first time, the two hPRL analogs G129 R-hPRL and S179D–hPRL have been synthesized in CHO cells in their authentic form with the properly folded molecular structure. For this purpose, the mammalian cell expression vector p658-hPRL, already generated in a previous work [35], was used, confirming its capability of rapidly and efficiently providing high expression levels of the proteins of interest, via a simple one-step amplification procedure. Routine production of G129R–hPRL in bacteria has been previously reported in several publications [18,19,26,45]. In these reports, the production yields were similar to those obtained for WT hPRL, which is in good agreement with this study, since we did not face any problem for producing or purifying this analog using either prokaryotic (data not shown) or eukaryotic recombinant systems. In another study performed by Chen and co-workers [20], G129R–hPRL was also synthesized in mammalian cells (mouse L-cells) but these authors described neither its purification nor its physico-chemical characterization, which precludes direct comparison with our study. This type of cells,

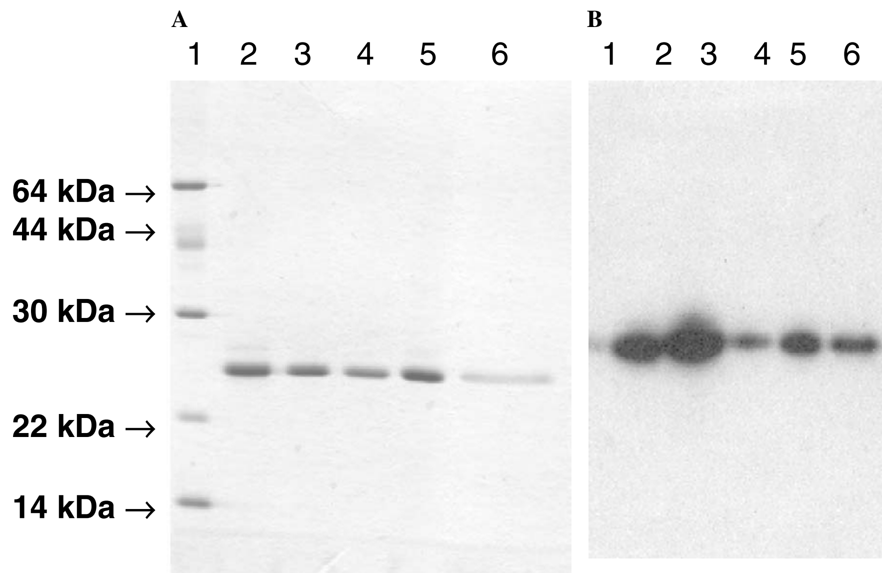

Fig. 5. SDS–PAGE (A) and Western blot (B) analysis under non-reducing conditions of CHO-secreted and purified hPRL, G129R–hPRL and S179D–hPRL. Lane 1, molecular mass markers. Lane 2, *E. coli*-secreted hPRL. Lane 3, CHO-secreted hPRL. Lane 4, *E. coli*-secreted G129R–hPRL. Lane 5, CHO-secreted G129R–hPRL. Lane 6, CHO-secreted S179D–hPRL.

Table 2
Retention times relative to hPRL (*E. coli*) obtained by HPSEC and RP–HPLC for the two analogs G129R–hPRL and S179D–hPRL and for hPRL (CHO)

Preparation	$t_{RR} \pm$ SD[a]	RSD[b] (%)
A. Obtained by HPSEC		
hPRL (CHO)	1.003 ± 0.0058	0.58
G129R–hPRL (*E. coli*)	1.003 ± 0.0058	0.58
G129R–hPRL (CHO)	1.013 ± 0.0057	0.57
S179D–hPRL (CHO)	0.997 ± 0.0058	0.58
B. Obtained by RP–HPLC		
hPRL (CHO)	0.99 ± 0.00019	0.02
G129R–hPRL (*E. coli*)	1.09 ± 0.015	1.40
G129R–hPRL (CHO)	1.10 ± 0.013	1.20
S179D–hPRL (CHO)	0.85 ± 0.010	1.20

[a] Median ± standard deviation, each value being the average of $n = 3$ determinations.
[b] Relative standard deviation expressed as percentage of the mean.

moreover, would be far from ideal for pharmaceutical production, especially when compared to CHO cells. The latter in fact, among the mammalian cell lines, are certainly the most productive, readily transfectable, capable of high-level gene amplification, easy to culture and hence are the most widely employed for manufacturing purposes [46].

The situation is different for S179D–hPRL analog. Indeed, all attempts to obtain significant production yields in bacterial periplasm were totally unsuccessful, providing at best expression levels <0.1 μg/mL/A_{600} by far lower than for G129R–hPRL (data not shown). This is reminiscent of a previous report suggesting that this analog preferentially refolds as multimers or aggregates after solubilization of inclusion bodies [26], leading to the recovery of much lower amounts of monomeric protein compared to WT hPRL or G129R analog. In contrast, we were able to produce this analog with a yield comparable, though lower, to that of other hPRLs, suggesting that the CHO system is certainly one of the best ways for obtaining the authentic form of S179D–hPRL.

A study of the best medium for CHO culture and protein production resulted in the choice of the chemically defined CD–CHO medium, since this provided the product

Table 3
Relative molecular masses (M_r) of hPRL, G129R–hPRL, and S179D–hPRL determined by MALDI–TOF mass spectrometry, in comparison with the theoretical values

Preparation	M_r	Theoretical difference from hPRL calculated value (%)	Difference between experimental and theoretical value (%)
hPRL (calculated theoretical value)	22898		—
rec-hPRL—*E. Coli*	22888		−0.04
rec-hPRL—CHO	22927		+0.13
G129R–hPRL (calculated theoretical value)	22997	+0.4	
G129R–hPRL—*E. coli*	23011[a]		+0.06
G129R–hPRL—CHO	23016		+0.08
S179D–hPRL (calculated theoretical value)	22926	+0.12	
S179D–hPRL—CHO	22943[a]		+0.07

[a] These values represent the average of $n = 2$ determinations, RSD being 0.015% and 0.027% for G129R–hPRL and S179D–hPRL, respectively.

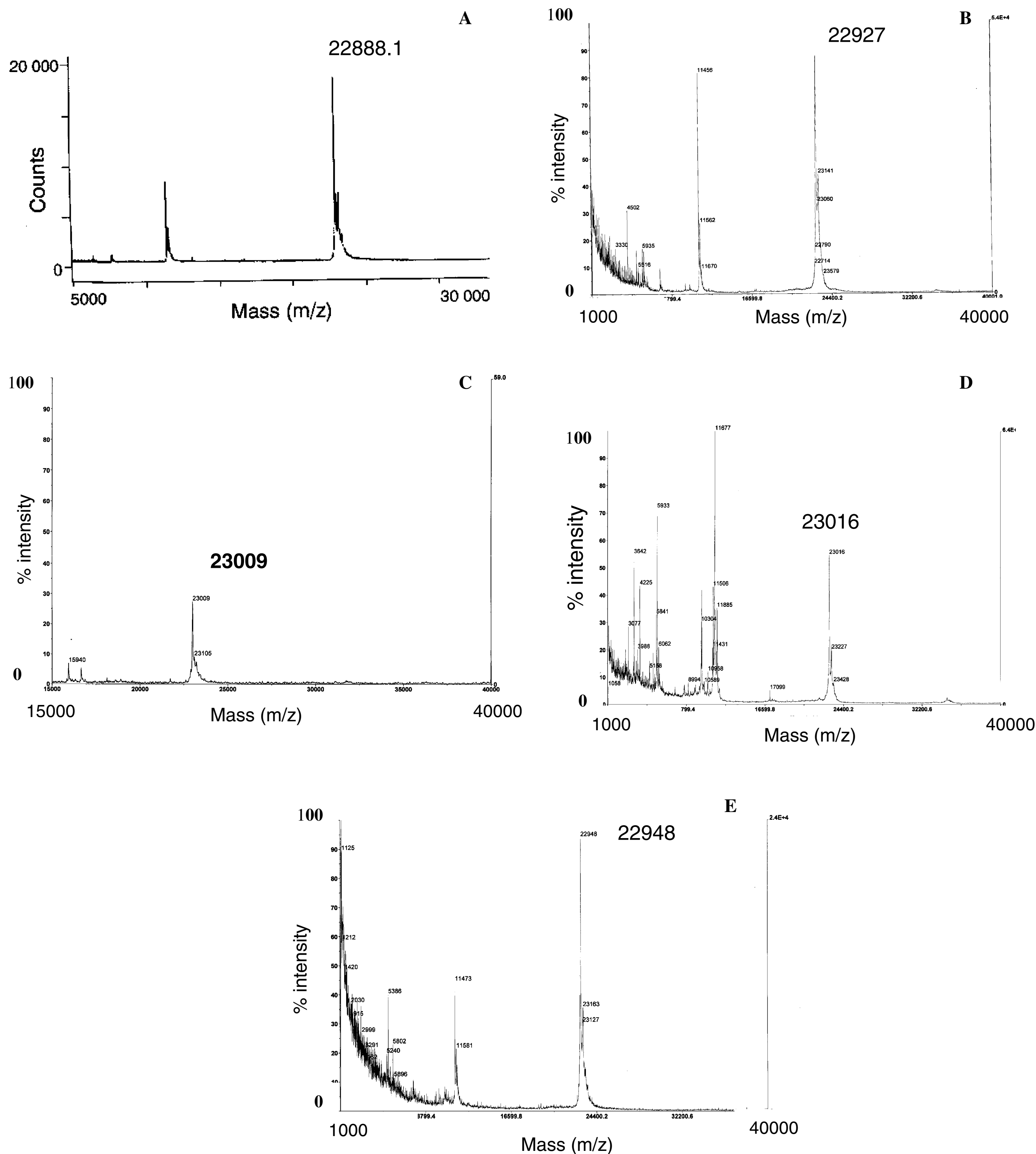

Fig. 6. MALDI–TOF–MS analyses of *E. coli*-secreted hPRL (A), CHO-secreted hPRL (B), *E. coli*-secreted G129R–hPRL (C), CHO-secreted G129R–hPRL (D), and CHO-secreted S179D–hPRL (E).

with the highest electrophoretic purity. This may also be due to the fact that, according to the manufacturer, this medium contains no proteins or peptide components of animal, plant or synthetic origin. A low level of contaminant proteins is obviously an important feature for facilitating subsequent purification.

The purification process was not designed with production in mind, but rather for readily obtaining sufficiently pure material suitable for proper characterization. The same purification protocol was applied to hPRL, G129R–hPRL and S179D–hPRL, thus permitting unbiased comparisons of these proteins in the various tests, a condition

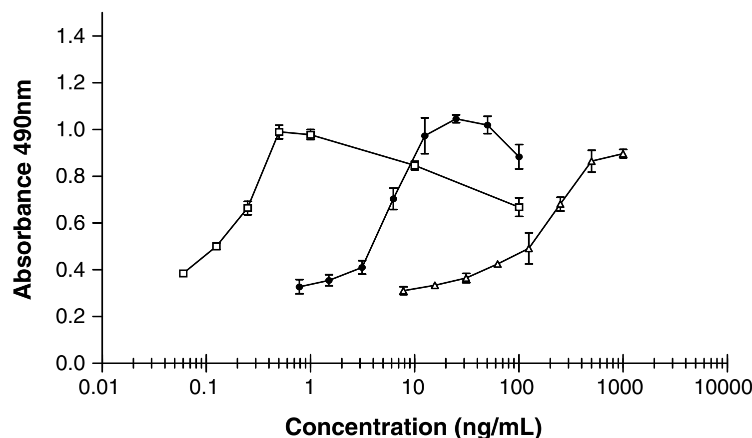

Fig. 7. Example of Ba/F3-LLP proliferative assay comparing the activities of WT-hPRL (□), G129R–hPRL (△), and S179D–hPRL (●).

Table 4
Ba/F3-LLP proliferative activity of the two analogs G129R–hPRL and S179D–hPRL, relative to the Chemical reference standard (CRS-WHO) of recombinant hPRL

Preparation	Slope ($A_{490\,nm}$ mL/ng) ± RSD[a] (%)	Relative potency
(A) On the basis of the slope of the dose–response curve		
hPRL	1.395 ± 15.7	—
S179D–hPRL	0.074 ± 18.9	53×10^{-3}
G129R–hPRL	0.001 ± 6.2	70×10^{-5}
Preparation	ED_{50} (ng/mL) ± RSD (%) [a]	Relative potency
(B) On the basis of ED_{50} of the dose–response curve		
hPRL	0.26 ± 18.1	—
S179D–hPRL	4.43 ± 33.8	59×10^{-3}
G129R–hPRL	202 ± 10.0	130×10^{-5}

[a] RSD, relative standard deviation, expressed as a percentage of the mean, each value being the average of $n = 3$ independent assays.

Table 5
Comparison between the Ba/F3-LLP proliferative activity of the two analogs G129R–hPRL and S179D–hPRL obtained in CHO cell conditioned medium and in *E. coli* cytoplasm.

Preparation	Slope[a] ($A_{490\,nm}$ mL/ng) ± RSD (%)	Relative potency
S179D–hPRL (CHO)	0.106 ± 16.0	—
S179D–hPRL (*E. coli*)	0.110 ± 14.6	1.04
G129R–hPRL (CHO)	0.0012 ± 27.0	—
G129R–hPRL (*E. coli*)	0.0011 ± 21.0	0.92

[a] Each value represents the average of $n = 3$ independent assays.

especially important for the biological assay. As already described for other biosynthetic hormones [35,41,44,47–49], RP–HPLC and HPSEC were utilized to follow the synthesis and purification steps. These two HPLC modes can, in fact, provide an early estimate of the secretion/purification yields and of the level of purity already attained. This is well-illustrated for G129R–hPRL in the RP–HPLC/HPSEC analysis that led to the selection of fraction #29 eluted from the SP–Sepharose fast flow column (Fig. 3). In this step, RP–HPLC is the most suitable for quantification, since it perfectly resolves the protein of interest from the bulk of impurities; HPSEC, on the other hand, can identify contaminants of different molecular size that can be more easily eliminated by preparative size-exclusion chromatography. In the case of fraction #29, HPSEC also permits a correct quantification because the G129R–hPRL peak is almost completely resolved from other contaminants. The peak area determined by HPSEC was, however, about 60% of that obtained by RP–HPLC, suggesting the presence of oligomeric or polymeric forms, which usually have the same hydrophobicity. Quite rapid and efficient chromatographic steps could be carried out by employing the long (60 cm) Tosohaas G2000 SW column in an analytical and preparative way. Thus, it was possible to obtain a product with >95% purity and analyze it in approximately 40 min.

As expected, no difference could be found between hPRL, G129R–hPRL and S179D–hPRL, by either SDS–PAGE/WB or HPSEC analysis, given that differences of the order of 0.12–0.4% in molecular mass (see Table 3) cannot be detected by these techniques. By RP–HPLC, however, it was possible to detect significant, previously unreported differences in hydrophobicity (11–14%) between hPRL and its two analogs. This suggests that a RP–HPLC–related hydrophobicity index might thus provide an interesting identity parameter, capable of rapidly discriminating the three molecular forms, their hydrophobic strength being G129R–hPRL> hPRL>S179D–hPRL. Since both substitutions are based on the shifting from a nonpolar or polar uncharged to charged amino acid, the hydrophobicity increase observed with G129R–hPRL cannot be attributed to the amino acid itself. As a matter of fact, in view of the extent of both hydrophobicity changes, they seem to be more a consequence of secondary or tertiary structure alterations. Considering the previously mentioned difficulties in obtaining significant amounts of S179D–hPRL in bacterial periplasm we could speculate that the described decrease of hydrophobicity of this molecule could be responsible for folding problems

and consequent multimerization and aggregation in the bacterial periplasmic environment.

Since the pioneering work of Karas and Hillenkamp [50], MALDI–TOF–MS analysis has permitted, via the conversion of large macromolecules into intact gas-phase molecular ions, accurate determination of the molecular mass of proteins. Application of this technique to human pituitary hormones is relatively recent [30,48,51–54] and has provided accurate and unequivocal determinations of the mass of glycoprotein hormones and their subunits [44]. Wu and co-workers [29], for example, employed MALDI–TOF–MS analysis to assess the phosphorylation status of human PRL extracted from pituitaries by identifying mono and diphosphorylated forms in NIDDK standard hPRL (i.e. revealing differences of the order of 80 daltons or 0.35%), with $\pm$0.1% accuracy. In the present work, MALDI–TOF–MS determinations proved to be extremely precise and accurate, the average difference in mass between preparations of hPRL or G129R–hPRL of different origins (*E. coli*- or CHO-derived) being of the order of 0.1%, and the mean mass difference between experimental and theoretical values for hPRL, G129R–hPRL and S179D–hPRL about 0.06%. The identity of the two analogs could thus be confirmed by this powerful tool that can discriminate between variants with extremely small mass differences.

Finally, purified proteins were characterized for their biological properties using the highly sensitive Ba/F3 LLP assay. This bioassay is an evolution of a new homologous assay recently developed by one of us for characterizing human lactogens [13]. This assay was initially developed by maintaining Ba/F3 cells stably expressing the human PRLR in culture medium containing 10 ng/mL of hPRL, which selected a cell sub-population (called Ba/F3 LP) exhibiting maximal growth at 10 ng/mL in proliferation assays [13]. In this study the concentration of hPRL in routine culture medium was dropped to 1 ng/mL, which further selected cells (called Ba/F3 LLP) able to grow in the presence of very low concentrations of lactogens, as confirmed by the dose–response curve obtained with WT hPRL in proliferation assays (Fig. 7). It is interesting to note that this new bioassay exhibits approximately the same sensitivity as the reference rat Nb2 assay, but presents the advantage of being mediated by a physiologically relevant hormone–receptor interaction for ligands of human origin. We would like to emphasize also that no significant difference has been observed between the biological activity of the analog obtained from bacterial cytoplasm and that synthesized in CHO cells. The synthesis of authentic proteins (i.e. without any initial methionine) is, in our opinion, always preferable, not only because it avoids time consuming procedures but also because it eliminates the potential immunogenicity due to the presence of this extra amino acid [46,55–57]. Also, even though the unnatural N-terminal methionine appears to have no effect on the biological activity of prolactin and many other molecules, an N-terminal methionine can drastically alter the bioactivity of some molecules. It has, for example, been reported that retention of the initiating methionine not only abrogates the physiological activity of the chemokine, RANTES, but also confers potent antagonist properties [58]. The effect of the methionine must therefore be considered when evaluating new products. In addition, the ability to produce a protein that is consistent from batch to batch is of significant relevance to future therapeutic production.

As previously reported, the apparent properties of PRL analogs are directly dependent on the sensitivity of the bioassay used [14]. The aim of using the highly sensitive Ba/F3 LLP bioassay in this study was thus to ensure the detection of any residual agonistic activity displayed by the two hPRL analogs analyzed, otherwise described as PRLR antagonists. In agreement with the results obtained using bacterially produced S179D–hPRL and G129R–hPRL [26], these analogs produced in CHO cells exhibit residual activity since both were able to stimulate Ba/F3 LLP cells to a detectable level. Both dose–response curves were shifted to the right, which presumably reflects, at least in part, the detrimental effect of these substitutions on global affinity for the receptor [18,26]. G129R–hPRL was the less potent, in agreement with the fact that this mutation, which affects binding site 2, strongly alters functional receptor dimerization [18,19,59]. In agreement with a further study [26], substitution of serine 179 did not completely abolish the mitogenic activity of PRL. This amino acid replacement appeared less detrimental to the bioactivity of PRL compared to the G129R mutation since the dose–response curve of the S179D mutant was displaced to the high concentrations by about 2 log units.

In conclusion the use of another recombinant expression system (CHO) and the new highly sensitive Ba/F3 LLP assay, confirms previous results obtained using bacterially produced hPRL analogs characterized in the less sensitive Ba/F3 LP bioassay. Altogether, these results indicate that, as recently discussed [14], both G129R–hPRL and S179D–hPRL should be viewed as weak agonists. Binding studies comparing G129R and S179D–hPRL to WT hPRL have already been published indicating 10-fold reduced affinity of the former [45] and 20-fold reduced affinity of the latter [26] towards the hPRLR. Weak agonists, however, can in some instance act as antagonists in the presence of the more growth-promoting hPRL and both have been demonstrated to have antagonist activity [31,32]. This is very similar to phytoestrogens and the widely used estrogen antagonist, tamoxifen, both of which are weak agonists when used alone, but effective antagonists in the presence of estrogen. Further development of PRL antagonists for therapeutic purposes has followed two very different approaches in two of our laboratories. In one case, effort has been focused on the development of analogs of hPRL devoid of any residual growth-promoting activity [14,22]. Such analogs (e.g. D1-9-G129R) could be used at high concentrations without fear of promoting PRL-dependent cell-growth. For this purpose, the Ba/F3 LP and LLP lines are ideal bioassays. In the other case, the ability of S179D–hPRL to inhibit signaling from hPRL and to produce an alternative intracellular signal has been further explored. Thus, S179D–hPRL has been shown

to inhibit Stat 5 signaling to cyclin D1 from hPRL [28], and to result in prolonged activation of ERKs 1 and 2 in cells expressing only the long hPRL receptor [60]. This prolonged activation of ERKs 1 and 2 leads to upregulation of the short form 1b of the hPRL receptor and subsequent amplification of ERK signaling [60], which in turn has been shown to result in increased expression of cell cycle regulatory proteins including p21 and the vitamin D receptor [60]. Therefore, the final effect of S179D–hPRL, such as long term antagonism of hPRL-promoted growth, is largely dependent on altered splicing of the hPRL receptor. Since altered splicing cannot be accomplished in the Ba/F3 LP and LLP lines, these are not good bioassay systems in which to analyze the antagonistic effects of S179D–hPRL. Both approaches to the development of therapeutically useful molecules have their merits. The first eliminates concerns over residual agonist activity regardless of dose. The second takes advantage of activity at the receptor which results in effective antagonism of hPRL. Results of this study clearly address aspects of the potential therapeutic use of these hPRL analogs as antagonists to the tumor-promoting effects of hPRL and thus their possible contribution to the treatment of human breast and prostate cancer.

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