Protein Engineering, Design & Selection, 2017, pp. 1–10
doi: 10.1093/protein/gzx062
Original Article

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Novel reagents for human prolactin research: large-scale preparation and characterization of prolactin receptor extracellular domain, non-pegylated and pegylated prolactin and prolactin receptor antagonist

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Received 26 October 2017; Revised 23 November 2017; Editorial Decision 28 November 2017; Accepted 30 November 2017

Abstract

To provide new tools for *in vitro* and *in vivo* prolactin (PRL) research, novel protocols for large-scale preparation of untagged human PRL (hPRL), a hPRL antagonist (del 1–9-G129R hPRL) that acts as a pure antagonist of hPRL in binding to hPRL receptor extracellular domain (hPRLR-ECD), and hPRLR-ECD are demonstrated. The interaction of del 1–9-G129R hPRL with hPRLR-ECD was demonstrated by competitive non-radioactive binding assay using biotinylated hPRL as the ligand and hPRLR-ECD as the receptor, by formation of stable 1:1 complexes with hPRLR-ECD under non-denaturing conditions using size-exclusion chromatography, and by surface plasmon resonance methodology. In all three types of experiments, the interaction of del 1–9-G129R hPRL was equal to that of unmodified hPRL. Del 1–9-G129R hPRL inhibited the hPRL-induced proliferation of Baf/LP cells stably expressing hPRLR. Overall, the biological properties of del 1–9-G129R hPRL prepared by the protocol described herein were similar to those of the antagonist prepared using the protocol reported in the original study; however, the newly described protocol improved yields by >6-fold. To provide long-lasting hPRL as a new reagent needed for *in vivo* experiments, we prepared its mono-pegylated analogue and found that pegylation lowers its biological activity in a homologous *in vitro* assay. As its future use will require the development of a PRL antagonist with highly elevated affinity, del 1–9-G129R hPRL was expressed on the surface of yeast cells. It retained its binding capacity for hPRLR-ECD, and this methodology was shown to be suitable for future development of high-affinity hPRL antagonists using a library of randomly mutated open reading frame of del 1–9-G129R hPRL and selecting high-affinity mutants by yeast surface display methodology.

Key words: homologous bioassay, prolactin, prolactin antagonist, prolactin receptor extracellular domain

Introduction

Human prolactin (hPRL), a hormone primarily secreted by the anterior pituitary gland, plays important roles in a variety of biological functions, exerting its effects on reproduction, growth, development, metabolism and immunomodulation (Sinha, 1995; Freeman *et al.*, 2000; Goffin *et al.*, 2002; Ben-Jonathan *et al.*, 2008). Excess local or systemic PRL levels have been reported to promote prostate cancer, gastrointestinal cancer and hematopoietic cancer (Goffin and Touraine, 2015). Human PRL acts by binding PRL receptor (PRLR) that is composed of extracellular and intercellular domains connected by a single transmembrane domain (Boutin *et al.*, 1988; Broutin *et al.*, 2010).

The active hPRL–hPRLR complex involves the formation of an asymmetric trimer that includes one ligand and two receptor moieties (Broutin *et al.*, 2010). The stabilization of this complex involves three intermolecular interactions: PRL site 1 interacts with one PRLR moiety, PRL site 2 interacts with the second PRLR moiety and the two PRLR extracellular domains (PRLR-ECDs) interact with each other (site 3) (Goffin *et al.*, 2005; Broutin *et al.*, 2010; Brooks, 2012). Suitable orientation of this asymmetric complex induced by hPRL binding leads to activation of receptor-associated kinases that trigger intercellular signaling pathways, e.g. JAK2/STATs, ERK1/2 and AKT (Brooks, 2012).

Given the increased evidence for PRLR signaling as a cancer promoter (for review, Goffin and Touraine, 2015; Goffin, 2017), a number of PRLR inhibitors (PRL analogues or anti-PRLR antibody) have been developed. All hPRL antagonists rely on steric hindrance within site 2 (Tallet *et al.*, 2008). This leads to a PRL variant that can bind to PRLR via site 1 but cannot induce the appropriate site-2 conformational changes to elicit intracellular signaling. The pioneering and best-documented hPRL antagonist (G129R hPRL) was created by replacing a glycine at residue 129 in helix 3 with a more bulky and charged arginine (Goffin *et al.*, 1994, 1996; Bernichtein *et al.*, 2003a, 2003b; Jomain *et al.*, 2007). *In vitro*, G129R-hPRL binds a single PRLR-ECD with high affinity but is restricted from binding a second PRLR-ECD due to markedly altered site 2 affinity resulting from the G129R mutation (Jomain *et al.*, 2007). In cells, this translates into the inability of G129R-hPRL to induce the conformational changes that are necessary to shift the pre-formed PRLR dimer into an active heterotrimeric complex. Despite of the fact that G129R-hPRL displays antagonistic properties, it was shown to retain some residual activity (Goffin *et al.*, 1994; Bernichtein *et al.*, 2003a) that may be sufficient to overcome antagonism at concentrations normally required to bind and block all receptors. Additional deletion of the 9 N-terminal residues in G129R-hPRL successfully abolished residual agonism (Bernichtein *et al.*, 2003b). Like G129R-hPRL, the so-called del 1–9-G129R-hPRL exhibited ~10-fold lower binding affinity than unmodified human PRL in binding assay to intact homogenates of HL5 cells (293 HEK cells stably expressing the human PRLR) (Kinet *et al.*, 1999).

The first aim of the present work was to develop a protocol for large-scale preparation of several recombinant proteins of wide interest in the hPRL field, including hPRLR-ECD, hPRL and pegylated hPRL. Such large-scale preparations will allow not only *in vitro*, but also *in vivo* experiments to characterize long-lasting PRL action of hPRL and hPRL antagonist. In addition, we aimed to challenge a new protocol for refolding del 1–9-G129R-hPRL to test whether it might improve its binding affinity to hPRLR. Finally, as a first step for future affinity saturation of hPRL antagonists using yeast surface display, we tested whether del 1–9-G129R-hPRL expressed on the surface of yeast cells retains its ability to bind hPRLR-ECD.

Materials and Methods

Materials

Restriction enzymes were from Fermentas (Vilnius, Lithuania) and highly purified DNA primers from Syntezza (Jerusalem, Israel). Nalidixic acid, isopropyl β-D-thiogalactopyranoside (IPTG) and kanamycin were purchased from Sigma (St. Louis, MO). Superdex 75 HR 10/30 and 26/60, and 200 HR 10/30 columns, Q-Sepharose and SP-Sepharose were from GE Healthcare (Piscataway, NJ). Molecular markers for SDS-PAGE and Bradford protein assay were purchased from Bio-Rad (Hercules, CA). Bacto tryptone was from Conda Laboratories (Madrid, Spain) and Bacto yeast extract and Bacto casamino acids (-Trp and -Ura) were from Difco (BD Biosciences, Franklin Lakes, NJ). Sulfosuccinimidyl 6-(biotinamido)hexanoate (sulfo-NHS-LC biotin) was purchased from Pierce (Rockford, IL). Plasmid pCT302 and strain EBY100 of the yeast *Saccharomyces cerevisiae* were provided by Dr E. T. Boder from the University of Tennessee, Knoxville, TN. Anti-c-Myc tag-fluorescein isotchiocyanate (FITC) labeled mouse IgG was purchased from Thermo Fisher Scientific (Waltham, MA) and streptavidin phycoerythrin conjugate was from Pharmingen (San Jose, CA). Methoxy PEG-propionylaldehyde-20 kDa was purchased from Jenkem Technology Inc. (Allen, TX). Fetal bovine serum, penicillin/streptomycin (10 000 units/mL and 10 000 mg/mL), and enhanced chemiluminescence (ECL) reagent were from Biological Industries Ltd (Beit Haemek, Israel). RPMI-1640 medium was from Invitrogen (Carlsbad, CA), peroxidase-conjugated streptavidin was from Jackson ImmunoResearch (West Grove, PA), and 3,3′,5,5′-tetramethylbenzidine (TMB) was from Dako (DakoCytomation, Copenhagen, Denmark). Other reagents (Tris, cysteine, arginine, NaOH, HCl, Tween 20, ultra-pure urea and skim milk) were all of analytical grade. XL-1 Blue cells were from Stratagene (La Jolla, CA) and Qiagen miniprep and QIAquick gel extraction kits from Qiagen (Valencia, CA). Other reagents were prepared in our laboratory: LB media (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, sterilized), TB media (80 g/L tryptone, 160 g/L yeast extract, 33.3 g/L glycerol, sterilized), YPD media (10 g/L yeast extract, 20 g/L peptone, 20 g/L dextrose, sterilized), SD-CAA media (20 g/L dextrose, 6.7 g/L Difco yeast nitrogen base, 5 g/L Bacto casamino acids, 5.4 g/L Na_2HPO_4 and 8.56 g/L NaH_2PO_4, sterilized), SG-CAA media (as for SD-CAA, but with 20 g/L galactose instead of dextrose), FACS/PBS buffer (8 g/L NaCl, 0.2 g/L KCl, 1.44 g/L Na_2HPO_4, 0.24 g/L KH_2PO_4) adjusted to pH 7.4 and supplemented with 5% (*w/v*) bovine serum albumin and 0.05% (*w/v*) azide, and TN buffer for gel-filtration experiments (25 mM Tris–HCl, pH 8 or 9, containing 300 mM NaCl).

Preparation of bacterial expression plasmids encoding hPRLR-ECD, hPRL and hPRL antagonist

Complementary DNA of the hPRLR-ECD (NCBI GenBank accession no. NM000949), encoding a polypeptide beginning at nucleotide G29, the fourth amino acid (Gly) downstream of the putative signal peptide cleavage site, and extending to D226, the last amino acid before the transmembrane domain, was ordered from Entelechon GmbH (Regensburg, Germany). The template was digested with *Nde*I and *Hind*III and inserted into these sites of the prokaryotic expression vector pMON which expressed the protein following induction with nalidixic acid (0.05 mg/mL). The construct was transfected into *Escherichia coli* MON105 cells. The DNA template encoding the sequences of hPRL (NCBI GenBank accession no. GQ305133.1), optimized for bacterial codon usage but conserving the same amino acid sequence, was ordered from Entelechon GmbH

(Regensburg, Germany). The cDNA was digested with *Nde*I and *Hind*III, extracted and ligated into pET29a expression vector linearized with the same restriction enzymes. The construct was inserted into *E. coli* strain BL21(DE3) and plated on LB-agar plates containing 0.025 mg/mL kanamycin for plasmid selection. Synthetic cDNA encoding the sequence of PRL antagonist (del 1–9-G129R-hPRL) was prepared by substituting a single glycine residue at position 129 with an arginine and deleting the 9 N-terminal residues. The cDNA, modified to ensure better codon usage and expression in *E. coli*, was ordered from Hylabs (Rehovot, Israel). The cDNA of the hPRL antagonist in pUC57 was digested with *Nde*I and *Hind*III, extracted, and ligated into pMon3250 expression vector linearized with the same restriction enzymes. *Escherichia coli* MON105-competent cells were transformed with the new expression plasmid and plated on LB-agar plates containing 75 μg/mL spectinomycin for plasmid selection. Eight *E. coli* colonies were isolated and confirmed to contain the hPRL antagonist cDNA by digestion with *Nde*I/*Hind*III. Five of them were sequenced and showed no DNA sequence errors.

Expression, refolding and purification of hPRLR-ECD, hPRL and del 1–9-G129R hPRL

Human PRLR-ECD

The MON105 cells transfected with the expression plasmid encoding hPRLR-ECD were grown in 8 L TB media (500 mL in 2.5-L flasks) at 37°C to an A_{600} of 0.9, and nalidixic acid was then added to 50 μg/mL. Cells were grown for an additional 4 h and then collected by centrifugation and frozen. Inclusion bodies (IBs) for subsequent refolding of hPRLR-ECD and the other two proteins were prepared as described previously for mammalian leptins (Raver *et al.*, 2002) and frozen. To prepare hPRLR-ECD, the IBs were solubilized in 50 mL of 4.5 M urea and 40 mM Tris base containing 10 mM cysteine, adjusted to pH 11.3 with NaOH. After 2 h of stirring at 4°C, two volumes of ice-cold water were added and stirred for an additional 1.5 h. Then the solution was dialyzed against 10 L of 10 mM Tris–HCl, pH 9, for 48 h, with five or six external solution exchanges. The protein solution of hPRLR-ECD was loaded at 200 mL/h onto a Q-Sepharose column (2.6 × 7 cm) pre-equilibrated with 10 mM Tris–HCl and 0.2 M NaCl. The breakthrough fraction, which contained the hPRLR-ECD, was collected and concentrated to 1 mg protein/mL. Then 18-mL portions were applied to a preparative Superdex 75 column (26/60 cm) pre-equilibrated with TN buffer (pH 9). Fractions containing the monomeric protein, as determined by gel filtration on an analytical Superdex 75 HR 10/30 column, were pooled, dialyzed against $NaHCO_3$ to ensure a 2:1 protein-to-salt ratio, and lyophilized.

Human PRL

The transfected BL21(DE3) cells expressing hPRL were incubated in TB media by shaking at 200 rpm, 37°C to an A_{600} of 0.9, and then IPTG (final concentration 0.4 mM) was added. After 4 h, the cells were collected by centrifugation and frozen. IBs prepared from 12 L of *E. coli* culture according to the protocol described above were solubilized in 50 mL of 4.5 M urea and 40 mM Tris base containing 1 mM cysteine, and adjusted to pH 11.3 with NaOH. After 2 h of stirring at 4°C, three volumes of 0.67 M L-arginine were added and stirred for 1.5 h at 4°C. Dialysis, elution in a Q-Sepharose column and analysis in a preparative Superdex 75 column were carried out as described in Human PRLR-ECD.

Del 1–9-G129R hPRL

Recombinant del 1–9-G129R hPRL was expressed by transfected MON105 cells in 10 × 500 mL of TB media in 2.5-L flasks, shaken at 200 rpm, 37°C. After OD_{600} reached 0.9, expression was induced with nalidixic acid (50 μg/mL), and the bacteria were grown for an additional 4 h. Subsequently, IBs obtained from 5 L of bacterial culture were solubilized in 500 mL of 4.5 M urea and 40 mM Tris base containing 0.1 mM cysteine, adjusted to pH 11.3 with NaOH. After 2 h of stirring at 4°C, three volumes of 0.67 M arginine were added to a final concentration of 0.5 M and stirred for an additional 1.5 h. Then the solution was dialyzed against 10 L of 10 mM Tris–HCl, pH 9, for 60 h, with five or six external solution exchanges. The protein solution was then applied at maximal flow rate (400–500 mL/h) onto a DEAE-cellulose column (5-mL bead volume) pre-equilibrated with 10 mM Tris–HCl, pH 9. Elution was carried out using a discontinuous NaCl gradient in the same buffer (50, 100, 150 and 300 mM NaCl) and 40-mL fractions were collected. Protein concentration was determined at 280 nm and the monomer content was obtained by size-exclusion chromatography (SEC) on an analytical Superdex 75 HR 10/30. The monomer-containing fractions were pooled, dialyzed against $NaHCO_3$ to ensure a 4:1 protein-to-salt ratio, and lyophilized. To check whether alternative refolding of del 1–9-G129R hPRL affects its affinity toward PRLR, IBs prepared from 5 L of bacterial culture (see above) were dissolved in 1000 mL 0.2 M phosphate buffer pH 7, containing 8 M urea and 1% (*v*/*v*) 2-mercaptoethanol at ratio of ~10 mg protein to 100 mL buffer. The solution was heated at 55°C for 5 min and then incubated at room temperature for 2 h. Refolding was performed by dialysis (72 h, 4°C, four changes) against 10 L of 25 mM NH_4HCO_3, pH 8.6. The precipitate formed during dialysis was removed by centrifugation then the supernatant was loaded onto a Q-Sepharose column equilibrated in 25 mM NH_4HCO_3, pH 8.6. Del 1–9-G129R hPRL was eluted by discontinuous NaCl gradient (0–300 mM), and fractions containing monomer eluted with 100 mM NaCl, as determined by gel-filtration chromatography on a Superdex 75 column, were pooled, dialyzed against $NaHCO_3$ to ensure a 4:1 protein-to-salt ratio, and lyophilized.

Circular dichroism

Circular dichroism (CD) spectra were recorded using a J-810 spectropolarimeter (JASCO) in a 0.1-cm quartz cuvette for far-ultraviolet CD spectroscopy. Spectra were collected over 190–260 nm at 25°C. Lyophilized hPRL and del 1–9-G129R hPRL were dissolved in water, dialyzed against 50 mM phosphate buffer, pH 7.5, for 20 h, and adjusted to 50 μM concentration. The CD measurements were performed at 25.0°C as controlled by thermoelectric Peltier elements to an accuracy of 0.1°C. The CD spectra were measured in five repetitions, resulting in an averaged spectrum for each protein. Standard deviation of the averaged CD signal at 222 nm was in the 5% range. For secondary structure determination, the CD data were expressed in degree × cm^2/dmol per mean residue, based on respective molecular mass. For secondary structure analysis, the DICHROWEB program was used and the α-helical content was calculated using the analytical program SELCON3.

Determination of purity and monomer content

SDS-PAGE was carried out according to Laemmli (1970) in a 15% polyacrylamide gel under reducing and non-reducing conditions. The gel was stained with Coomassie Brilliant Blue R. SEC was performed on a Superdex 75 HR 10/30 column for determination of hPRLR-ECD, hPRL and del 1–9-G129R hPRL, and on a Superdex

200 HR 10/30 column for pegylated hPRL. The column was pre-equilibrated and developed using TN buffer (pH 8) at room temperature. The columns were calibrated with leptin (16 kDa), human growth hormone (21.5 kDa), albumin (66 kDa), and bovine IgG monomer (150 kDa) and dimer (300 kDa).

Pegylation of hPRL

Methoxy PEG-propionylaldehyde-20 kDa was used for pegylation under conditions that favor pegylation of the N-terminal group. The hPRL (50 mg) was dissolved in 25 mL 1 M NaH_2PO_4 buffer, pH 6.5; then 425 µL of 1 M $NaBH_3CN$ was added, and the dissolved protein was conjugated with 12-fold molar excess of PEG dissolved in 10 mM HCl. The solution was stirred for 24 h and subsequently dialyzed against 50 mM NaH_2PO_4, pH 6.5. Then ammonium sulfate was added to 0.8 M and the solution was applied on a phenyl Sepharose column (20-mL bead volume) pre-equilibrated with 50 mM NaH_2PO_4 containing 0.8 M ammonium sulfate, pH 6.5. The pegylated proteins were eluted in 50 mM NaH_2PO_4, pH 6.5, containing, consecutively, 0.4 and 0.2 or 0 M $(NH_4)_2SO_4$. Fractions containing the mono-pegylated protein, as determined by SEC on an analytical Superdex 200 column, were pooled, dialyzed against $NaHCO_3$ to ensure a 2:1 protein-to-salt ratio, and lyophilized.

Biotinylation of hPRL and hPRLR-ECD

Human PRL (1 mg/mL) and hPRLR-ECD (1 mg/mL) were dissolved in PBS, pH 7, and incubated with a 10-fold molar excess of the biotinylation reagent sulfo-NHS-LC biotin for 40 min at room temperature. Excess non-reacted biotin was removed by dialyzing against PBS buffer.

Receptor binding assay

In all competitive-binding experiments, the immobilized hPRLR-ECD was used as the receptor, the biotinylated hPRL served as a ligand that could be competed off by either hPRL, PEG-hPRL or del 1–9-G129R hPRL. Polystyrene 96-well microtiter plates were coated overnight at 4°C with 100 µL of 40 µM hPRLR-ECD in PBS, pH 7.4. Wells were then washed once with PBST (PBS containing 0.05% *w/v* Tween 80) and blocked with PBS containing 3% (*w/v*) skim milk for 2 h at room temperature. All further incubations were carried out at room temperature. Wells were washed again once with PBST and incubated with different concentrations of unlabeled hPRL and hPRL antagonist (50 µL/well) for 30 min, and then 50 µL of 42 pM biotinylated hPRL was added to each well for another 2 h at room temperature. The wells were then washed three times with PBST and incubated with 1:10 000-diluted peroxidase-conjugated streptavidin for 1 h. Wells were washed three times with PBST, and then developed using 100 µL/well TMB peroxidase substrate (KPL, Zotal, Israel) diluted 1:4 and incubated for 30 min. The reaction was stopped with 50 µL of 2 N H_2SO_4 and the absorbance was read at 450 nm by ELISA MicroPlate Reader ELx808 (Bio-Tek Instrument Inc., Winooski, VT).

Detection of hPRL, PEG-hPRL and del 1–09-G129R hPRL complex formation with hPRLR-ECD and complex stability by SEC

To characterize the binding stoichiometry between hPRL, PEG-hPRL or del 1–9-G129R hPRL and hPRLR-ECD, they were mixed in different molar ratios, incubated for 30 min at 4°C and then separated under non-denaturing conditions by SEC using an analytical Superdex 75 HR 10/30 column equilibrated with TN buffer (pH 9) at room temperature as described previously (Niv-Spector *et al.*, 2005). To evaluate the stability of each complex, the complex solution at 10 µM of each component was serially diluted and the retention time (RT) of each complex was determined by SEC.

Surface plasmon resonance

The binding affinities of hPRL, PEG-hPRL and del 1–9-G129R hPRL toward hPRLR-ECD were measured by surface plasmon resonance (SPR) on a Biacore T200 instrument using a CM-5 Biacore sensor chip (GE Healthcare Bio-Sciences AB). Human PRL, PEG-hPRL and hPRL antagonist were diluted in 100 mM CH_3COONa, pH 4.6, to a final concentration of 20 µg/mL in a total volume of 200 µL, and coupled using the standard Biacore amine chemistry protocol. The analyte, hPRLR-ECD, was injected in running buffer (PBS) at different concentrations (10, 14, 20, 25, 33 and 50 nM) at a flow rate of 20 µL/min at 25°C. These conditions resulted in a linear relationship between the hPRL-ECD concentration and the maximal (steady-state) response, indicating a pseudo first-order regime in relation to the immobilized PRLs. Injections were performed simultaneously over two channels and a blank surface (channel 1) was used as the control. The net signal was obtained by subtracting the blank signal from the signal of the immobilized surface. The association phase for the binding was followed for 4 min, and the dissociation phases were monitored for 3 min. Surface regeneration between consecutive binding cycles included a 1-min injection of 2 mM NaOH. The response was monitored as a function of time (sensorgram) at 25°C. The resultant binding curves were fitted to the association and dissociation phases at all hPRLR-ECD concentrations simultaneously, using Biacore evaluation software.

Nb2-11C and BAF/LP cell-proliferation assays

Biological activities of hPRL and PEG-hPRL were estimated by calculating the proliferation rate of Nb2-11C cells expressing the short form of rat PRLR as described previously (Gertler *et al.*, 1985; Ocłoń *et al.*, 2017). Briefly, Nb2-11C cells were grown as a suspension culture in 75-cm^2 tissue-culture flasks (Nunc, Kamstrup, Roskilde, Denmark) in RPMI-1640 medium containing 5% (*v/v*) fetal calf serum (FCS) supplemented with antibiotic–antimycotic solution (10 000 units/mL penicillin, 10 mg/mL streptomycin and 25 µg/mL amphotericin B per mL). Stationary cultures were obtained by transferring the Nb2-11C cells into lactogen-free medium in which FCS was replaced with 5% (*v/v*) horse geld serum. The experiment was performed in 96-well plates seeded with 2.5×10^4 cell/well in 100 µL lactogen-free medium Human PRL (10 µL of 50, 12.5, 3.125, 0.781, 0.195 and 0.045 µg/mL) was added per well. The activity of PEG-hPRL was determined similarly, using, respectively, 10-fold higher concentrations (500–0.45 µg/mL). The cells were cultured in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. Cell number was assessed 48 h after hormone additions by 3-(4,5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) assay.

The proliferation rate of PRL-sensitive BAF/LP cells stably transfected with hPRLR was used to estimate the antagonistic activity of del 1–9-G129R hPRL as described previously (Ocłoń *et al.*, 2017). To determine antagonistic activity, 300 ng of wild-type (WT) hPRL was added to each well, which also contained different concentrations (600, 200, 66, 22, 7 and 2 µg/mL) of del 1–9-G129R hPRL antagonists prepared by two different refolding protocols. The average absorbance in wells without hPRL (negative control) was used as a blank value and subtracted from the other absorbance values to

yield corrected absorbance values. The average absorbance in wells with WT hPRL after subtracting the negative control was used as a positive control to calculate percent inhibition. The inhibition curves were drawn using Prisma (4.0) nonlinear regression sigmoidal one-site competition program, and the IC_{50} values were calculated.

Expression of active del 1–9-G129R hPRL on yeast cell surface

Expression plasmid and yeast cells expressing hPRL antagonist on their surface were prepared by a methodology used previously in our laboratory (Shpilman *et al*., 2011; Boder and Wittrup, 2000). Human PRL antagonist-encoding cDNA was modified by PCR to introduce *Nhe*I and *BamH*I restriction sites at the 5′ and 3′ ends, respectively, enabling subsequent subcloning into acceptor vector pCT302 linearized with *Nhe*I and *BamH*I. The primers used for the PCR were (forward) 5′-GTACGCAAGCTAGCGCTGTTCCGATCCAGAAAGTTCAGG-3′ and (reverse) 5′-CGTAGGATCCGCATTCCGGAGAAACGT CCAA CTG-3′. Del 1–9-G129R hPRL was displayed on the surface of yeast as a protein fused to the Aga2p mating agglutinin protein followed by a small DNA fragment encoding c-Myc. The Aga2p protein is tethered in the cell wall via disulfide bridges to the Agalp protein, in yeast strain EBY100 in which Agalp expression is inducible from a single, integrated open reading frame downstream of the GAL1-10 promoter (Shpilman *et al*., 2011). The presence of the fusion protein on the cell surface was detected by binding of biotinylated hPRLR-ECD and the proper in-frame location by detection of c-Myc using fluorescence-activated cell sorting (FACS).

The interaction of the surface-displayed hPRL antagonist flanked at the 3′ end by c-Myc with biotinylated hPRLR-ECD was determined in situ on the cell wall of whole cells via flow cytometry. One colony of *S. cerevisiae* strain EBY100 constructed to express del 1–9-G129R hPRL was taken from an SD-CAA agar plate and grown with shaking in SD-CAA media at 33°C for 24 h. Cells were then diluted to an OD_{600} of 1 (corresponding to ~10^7 cell/mL) and were induced for 22 h by exchanging the media with SG-CAA. Then, 3×10^6 yeast cells were aliquoted per tube and washed twice with 500 µL and 1000 µL cold FACS buffer (PBS, 0.05% *w/v* NaN_3, 5% *v/v* FCS) and resuspended in 500 µL FACS buffer with either 100, 20 or 4 nM biotinalyted hPRLR-ECD for 1 h with weak shaking at 4°C. The yeast cells were then washed again and incubated at 4°C in FACS buffer with 25 µL streptavidin conjugated to (*R*)-phycoerythrin (PE) (1:50) to detect expression of the biotinalyted hPRLR-ECD bound to hPRL. Simultaneously, the cells were incubated with 10 µL anti-c-Myc-FITC monoclonal antibody 9e10 (at 1:100) conjugated to fluorescein, to detect expression of the C-terminal c-Myc epitope tag. Then the cells were washed twice with 1 mL FACS buffer, once with 1 mL PBS and resuspended in 500 µL PBS for FACS. Flow cytometry analysis was performed on a BD FACSAria Fusion instrument (BD Immunocytometry Systems) equipped with 488-, 405-, 561- and 640-nm lasers, using a 100-µm nozzle and calibrated for single yeast cell detection, controlled by BD FACS Diva software v8.0.1 (BD Biosciences), at The Weizmann Institute of Science's Flow Cytometry Core Facility. Further analysis was performed using FlowJo software v10.2 (Tree Star). Subpopulations expressing both hPRL antagonist and c-Myc were detected using anti-c-Myc-FITC, biotinylated hPRLR-ECD and Strep–PE. FITC was detected by excitation at 488 nm and collection of emission using 502 longpass (LP) and 530/30 bandpass (BP) filters. PE was detected by excitation at 561 nm and collection of emission using a 582/15 BP filter. Flow cytometry analysis was performed on a BD FACSAria Fusion instrument (BD Immunocytometry Systems) equipped with a 488, 405, 561 and 640 nm lasers, using a 100 µm nozzle and calibrated for yeast single cell detection, controlled by BD FACS Diva software v8.0.1 (BD Biosciences), at The Weizmann Institute of Science Flow Cytometry Core Facility. Further analysis was performed using FlowJo software v10.2 (Tree Star).

Results

Purification and physicochemical characterization of hPRLR-ECD, WT PRL, del 1–9 G129R hPRL and pegylated hPRL

The refolded WT hPRL and hPRLR-ECD were purified in two steps on a Q-Sepharose column pre-equilibrated with 10 mM Tris–HCl and 0.2 M NaCl, and a preparative Superdex 75 column, whereas the refolded del 1–9-G129R hPRL was purified on a DEAE column by stepwise elution with increasing concentrations of NaCl. The del 1–9-G129R hPRL fractions eluted by 50 mM NaCl, as well as those of the other two proteins, were pure, as documented by SDS-PAGE carried out under reducing conditions (Fig. 1, lanes 2, 4 and 5) and by SEC analysis using a Superdex 75 HR 10/30 analytical column (Fig. 2). The proteins of interest consisted of >95% monomers eluted at RTs of 16.49 min (hPRL), 16.57 min (del 1–9-G129 hPRL antagonist) and 15.47 min (hPRLR-ECD) (Fig. 2A–C). The overall average yields of pure protein calculated per 5 L fermentation culture were 320 mg (hPRL), 206 mg (del 1–9-G129R hPRL) and 155 mg (hPRLR-ECD). Protein concentrations were calculated from their absorbance at 280 nm using DNAman program yielding respectively the following values for 1 mg/mL solutions at pH 8: WT hPRL and pegylated hPRL—0.92, 1–9-G129R hPRL—0.96 and hPRLR-ECD—2.63. To assess the activity of the previously reported del 1–9-G129R hPRL (Bernichtein *et al*., 2003a), the antagonist was refolded from the same IBs using an alternative protocol. The purity, as determined by SDS-PAGE and analytical SEC, was identical to that prepared by the present protocol (not shown), but the yield was only 35 mg from 5 L fermentation culture. Pegylation of hPRL resulted in a yield of 15.5 mg PEG-hPRL from 50 mg of non-pegylated PRL. The purity and homogeneity of the PEG-hPRL was documented by SDS-PAGE (Fig. 1, lane 1), with the main band appearing at ~45 kDa, as expected. A small amount of double-pegylated hPRL, which can be seen as a weak band at ~75 kDa, was also detected. Similarly, the pegylation reaction monitored on a

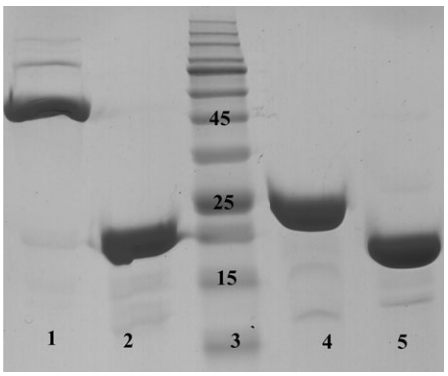


Fig. 1 SDS-PAGE analysis of hPRL, PEG-hPRL, del 1–9-G129R hPRL and hPRLR-ECD on a 15% gel. Lanes: 1, PEG-hPRL; 2, hPRL; 3, molecular mass markers in kDa; 4, hPRLR-ECD; 5, del 1–9-G129R hPRL.

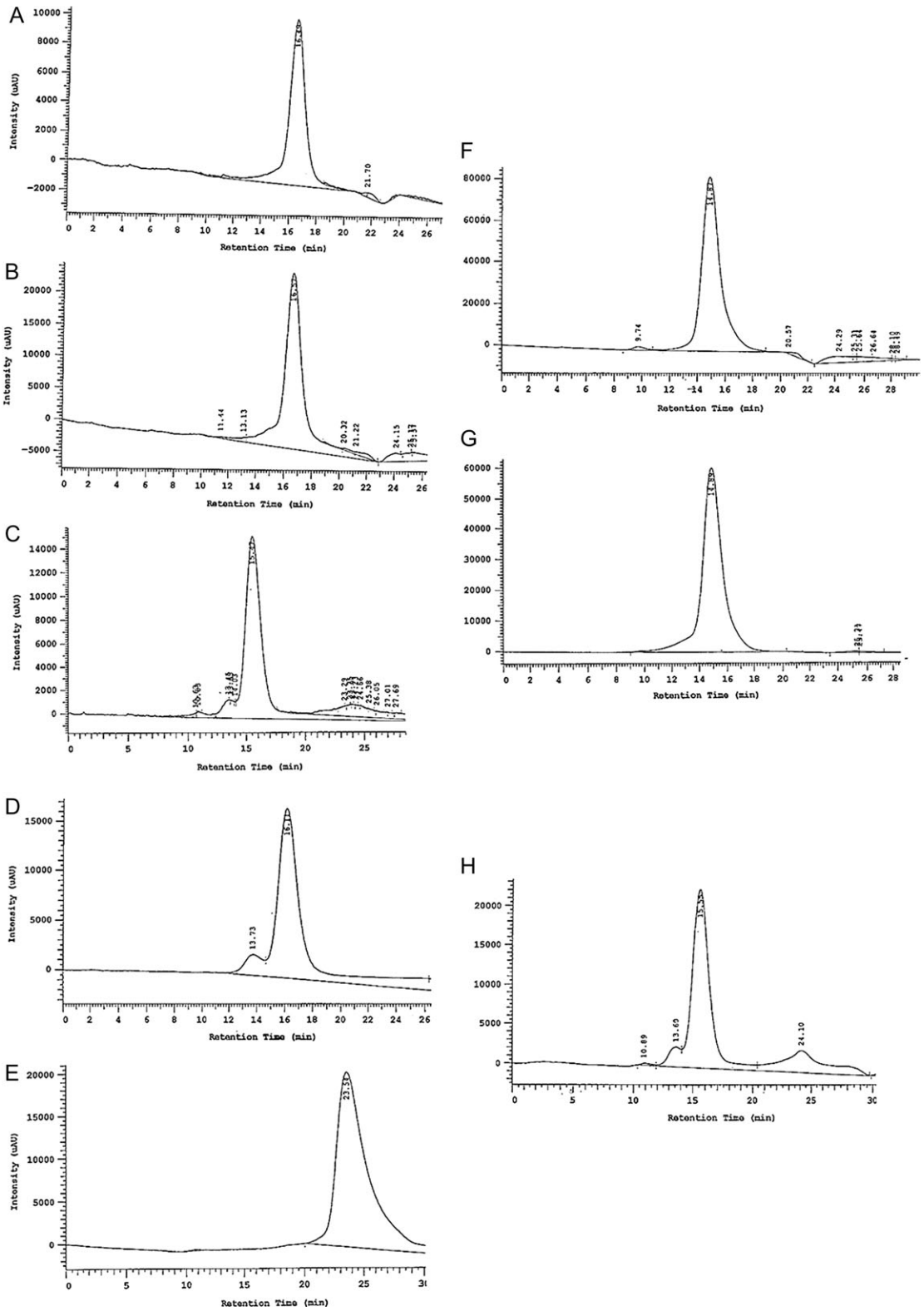


Fig. 2 SEC of purified proteins and their complexes with hPRLR-ECD. hPRL (**A**), del 1–9-G129R hPRL (**B**), hPRLR-ECD (**C**), PEG-hPRL (**D**), hPRLR-ECD (**E**), hPRL–hPRLR-ECD complex (**F**), del 1–9-G129R hPRL–hPRLR-ECD complex (**G**), PEG-hPRL–hPRLR-ECD complex (**H**). (A–C) and (F–G) were developed on Superdex HR 75 10/30 column in TN buffer pH 8 at 0.8 mL/min, and (D, E and H) on Superdex HR 200 10/30 column in TN buffer pH 8 at 0.7 mL/min. The separation was carried out at room temperature and the proteins were detected at 280 nm.

Superdex 200 HR 10/30 column exhibited > 90% mono-pegylated hPRL, with a RT of 16.11 min and ~9% double-pegylated hPRL, detected as a smaller peak with RT of 13.73 (Fig. 2D). Calibration of the column with reference proteins indicated a RT of 16.11 min, which corresponds to ~220 kDa due to highly increased hydrodynamic volume.

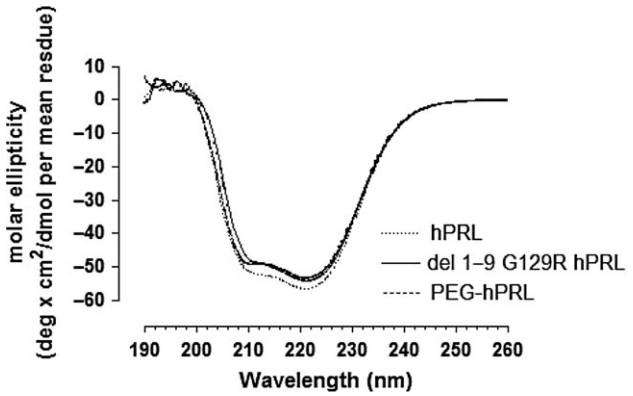


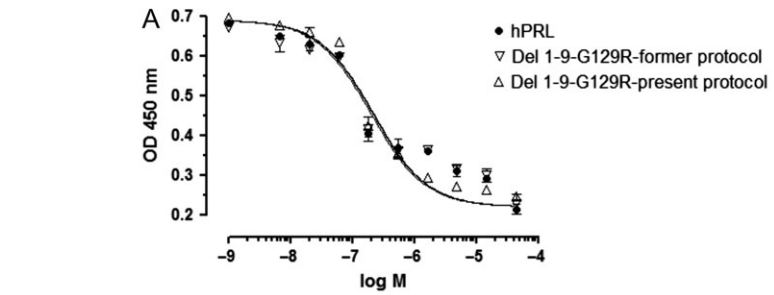
Fig. 3 Comparing the folding of the recombinant mutant and PEG-hPRL to hPRL. The CD spectra of purified recombinant hPRL, PEG-hPRL and del 1–9-G129R hPRL were collected over 190–260 nm at 25°C. Lyophilized hPRL and del 1–9-G129R hPRL were dissolved in water, dialyzed against 50 mM phosphate buffer, pH 7.5, for 20 h, and adjusted to 50 μM. For secondary structure analysis, the DICHROWEB program was used and the α-helical content was calculated using the analysis program SELCON3.

To characterize the folding propensity of our recombinant proteins, we performed CD analysis. CD spectra (Fig. 3) gave almost identical profiles for hPRL and del 1–9-G129R hPRL, and the percentages of α-helix were, respectively, 53% and 56%. The CD spectrum of PEG-hPRL was similar (56%), indicating that pegylation did not alter its secondary structure content. For calculation we used the programs of Sreerama and Woody(2000) and Whitmore and Wallace (2004).

Binding experiments

The binding activity of del 1–9-G129R hPRL was compared to that of hPRL in three types of experiments. First, in a competitive non-radioactive binding assay using biotinylated hPRL as the ligand and hPRLR-ECD as the receptor, both del 1–9-G129R hPRL and hPRL displaced the biotinylated hPRL with respective EC_{50} values of 2.0921×10^{-7} M and 1.9515×10^{-7} M, a non-significant difference (Fig. 4A). A comparison of the binding properties of del 1–9-G129R hPRL prepared by the two different refolding protocols (see Materials and Methods) showed no difference (Fig. 4A), nor was there any difference in binding between pegylated and non-pegylated hPRL (Fig. 4B).

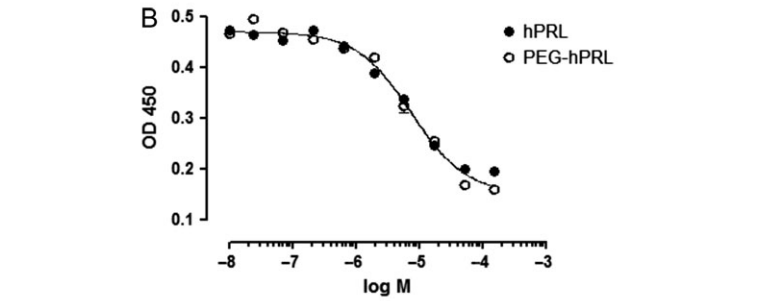
Secondly, the binding between soluble hPRLR-ECD and hPRL or del 1–9-G129R hPRL was also demonstrated by SEC analysis of the formed soluble complexes. Formation of 1:1 complex (involving site 1) between hPRL (RT = 16.49 min) or del 1–9-G129R hPRL



	hPRL	Del 1–9-G129R-former protocol	Del 1–9-G129R-present protocol
EC50	1.9515e–007	2.0921e–007	2.1469e–007

95% Confidence Intervals

	hPRL	Del 1–9-G129R-former protocol	Del 1–9-G129R-present protocol
EC50	1.3700e–007 to 2.7797e–007	1.4213e–007 to 3.0796e–007	1.7592e–007 to 2.6201e–007



	hPRL	PEG-hPRL
EC50	7.5458e–006	7.2659e–006

95% Confidence Intervals

	hPRL	PEG-hPRL
EC50	6.5862e–006 to 8.6452e–006	6.2719e–006 to 8.4173e–006

Fig. 4 Competitive displacement of biotinylated hPRL bound to plated hPRLR-ECD by hPRL or del 1–9-G129R hPRL prepared by two different refolding protocols (**A**), and by hPRL or PEG-hPRL (**B**). For other details, see text.

Table I. Calculation of kinetics and thermodynamic constants for the interaction of immobilized hPRL, PEG-hPRL and del 1–9-G129R hPRL with soluble hPRLR-ECD measured by SPR

Protein tested	k_{on} (m/s)	k_{off} (1/s)	Kd (M)	Ka (1/M)
hPRL	4.95×10^{4}	1.61×10^{-3}	3.25×10^{-8}	3.07×10^{7}
PEG-hPRL	4.24×10^{4}	1.47×10^{-3}	3.47×10^{-8}	2.89×10^{7}
Del 1–9-G129R hPRL	4.97×10^{4}	1.60×10^{-3}	3.22×10^{-8}	3.10×10^{7}

(RT = 16.57 min) and hPRLR-ECD (RT = 15.47 min) (Fig. 2A–C) was demonstrated by RTs of 14.87 and 14.85 min, respectively (Fig. 2F,G). To characterize the binding stoichiometry between PEG-hPRL and hPRLR-ECD, the experiment was performed using either a constant concentration (10 μM) of PEG-hPRL and increasing concentrations of hPRLR-ECD or vice versa. All components added alone were eluted from the Superdex 200 HR 10/30 column as monomers at RTs of 15.73 min (PEG-hPRL) and 23.54 min (hPRLR-ECD) (Fig. 2D,E). Mixing the two components in a 1:1 molar ratio resulted in a new single peak with RT of 15.55 for (PEG-hPRL–hPRLR-ECD) along with almost complete disappearance of free hPRLR-ECD (Fig. 2H). Using SEC analysis, we also compared the stability of the 1:1 hPRL–hPRLR-ECD complex to that of del 1–9-G129R hPRL–hPRLR-ECD at a 1:1 molar ratio. We employed SEC on an analytical Superdex HR 75 10/30 column, which determines the molecular mass of the complex under non-denaturing conditions. The experiments were performed using constant concentrations (10 μM) of both components as described above. Gradual dilution of the respective 1:1 complexes of hPRL or del 1–9-G129R hPRL and hPRLR-ECD from 10 to 0.2 μM changed the RT of the complex peak only slightly, from 14.85 to 14.97 min (not shown), indicating no difference between the two complexes and similar stability and affinity. It should be noted that during the SEC analysis, there is a further, up to 10-fold dilution of the complexes.

In the third experiment, a more quantitative characterization of the interaction of pegylated and non-pegylated hPRL and del 1-9-G129R hPRL with hPRLR-ECD was obtained using the SPR methodology. As shown in Table I, both kinetic and thermodynamic constants of the three tested proteins were almost identical. This experiment was repeated once more with similar results (not shown), clearly indicating that pegylated and non-pegylated hPRL and del 1–9-G129R hPRL have similar affinities to hPRLR-ECD.

Biological agonistic activity of non-pegylated and pegylated hPRL and antagonistic activity of del 1–9-G129R hPRL

The biological activity of non-pegylated and pegylated hPRL was tested in Nb2-11C cells (stably transfected with the short form of rat PRLR) using cell-proliferation rate as the readout. Results of a typical experiment (out of two), presented in Fig. 5A, showed that pegylation reduced reduces the mitogenic activity of hPRL ~7-fold. In a homologous bioassay in Baf/LP cells, the activity of pegylated hPRL was ~20-fold lower (Fig. 5B). The antagonistic activity of del 1–9-G129R hPRL was tested in Baf3/LP cells stably transfected with hPRLR. Data presented in Fig. 5C were obtained by stimulating cell proliferation with hPRL (300 ng per well) and inhibiting it in a dose-responsive manner with increasing concentrations of del 1–9-G129R hPRL prepared by two different refolding methods. There was no significant difference in antagonistic activity between the antagonists prepared by the different refolding protocols (Fig. 5C). Del 1–9-G129R hPRL did not exhibit any agonistic activity in Baf3/LP cells (not shown).

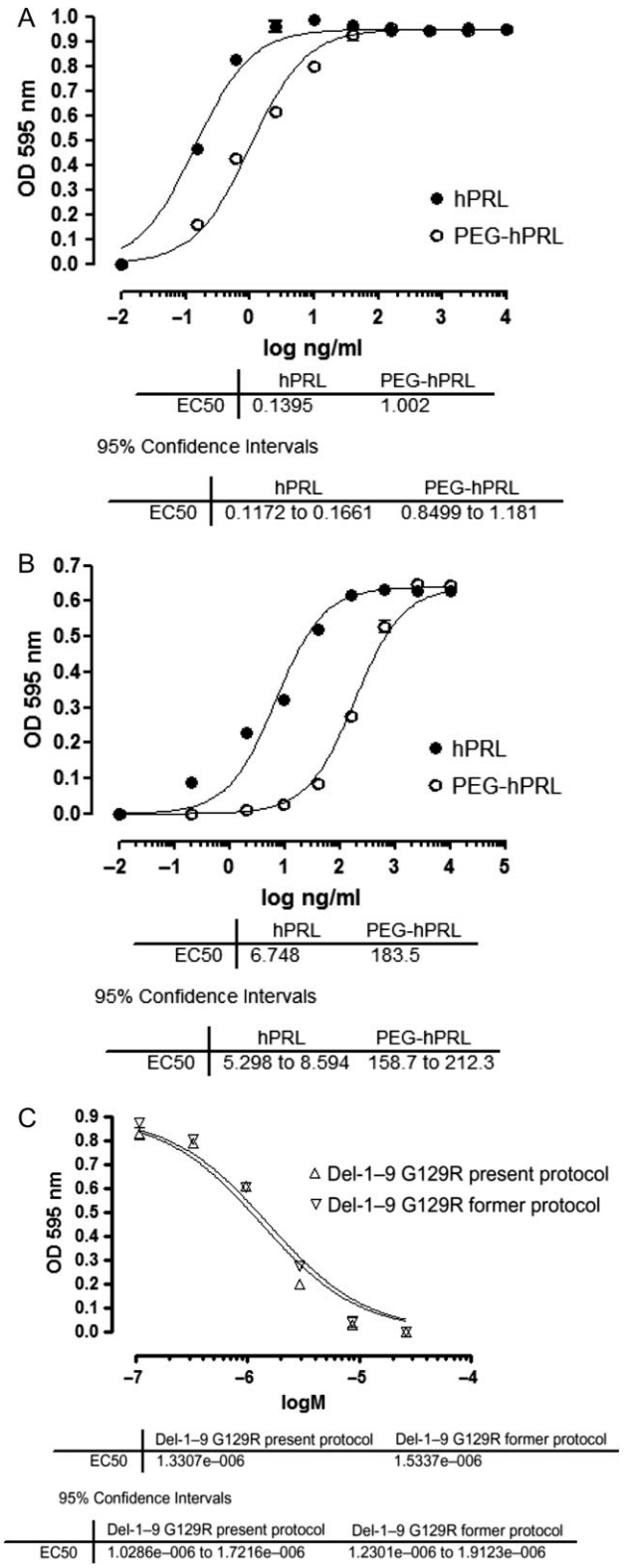


Fig. 5 Comparison of biological activity of pegylated and non-pegylated hPRL in Nb2-11C cells (**A**), Baf/LP cells (**B**), and antagonistic activity of del 1–9-G129R hPRL prepared by two different refolding protocols in Baf/LP cells (**C**).

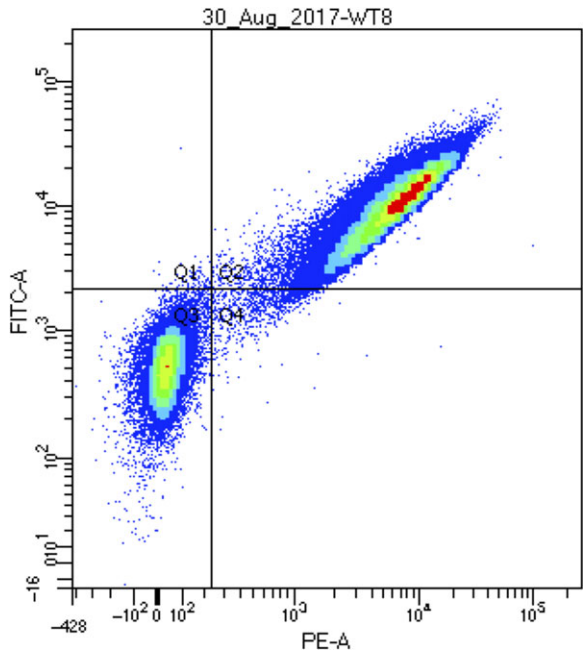


Fig. 6 Representative flow cytometry analysis demonstrating a proof of concept for quantifying the approximate affinity of yeast cell surface-displayed hPRL flanked at the C-terminal with c-Myc epitope tag to hPRLR-ECD. Cells were incubated with 100 nM biotinylated hPRLR-ECD, then with streptavidin conjugated to PE to detect the expression of the biotinalyted hPRLR-ECD bound to the hPRL. Simultaneously, the cells were incubated with anti-c-Myc-FITC monoclonal antibody to detect the expression of the C-terminal c-Myc epitope tag.

Detection of binding capacity of del 1–9-G129R hPRL expressed on the surface of yeast cells

As shown in Fig. 6, ~2/3 of the screened yeast cells reacted with both biotinylated hPRLR-ECD and anti-c-Myc antibody. This indicates that del 1–9-G129R hPRL expressed on the yeast cell surface retains its binding capacity.

Discussion

As possible future use of recombinant PRL antagonists for *in vivo* pharmacological intervention requires large amounts of recombinant protein, in the present work we developed new protocols for large-scale preparation of relevant proteins, such as hPRL, hPRL antagonist and hPRLR-ECD. We also documented that the inhibitory activity of a previously reported recombinant hPRL antagonist (Goffin *et al.*, 1994, 1996; Bernichtein *et al.*, 2003a, 2003b; Jomain *et al.*, 2007) is identical to that reported in the present protocol, which gives much higher yield (Fig. 5C). In agreement, binding to recombinant PRLR-ECD *in vitro* using three different experimental approaches that measure site 1-mediated interaction with the recombinant hPRLR-ECD showed no differences between hPRL and del 1–9-hPRL G129R, irrespective of the refolding protocol (Fig. 4A). This confirms that the 10-fold lower affinity of del 1–9-G129R hPRL that is measured on membrane-anchored PRLR (Bernichtein *et al.*, 2003a) is intrinsic to the antagonist and results from the mutations affecting site 2. As theoretical thermodynamic considerations indicate that if agonist and antagonist exhibit the same affinity, a 100-fold molar excess of antagonist should lead to 99% of the ligand-bound receptors being occupied by the latter. Thus, a 100-fold increase in antagonist affinity is expected to lead to similar results at ~1:1 antagonist:agonist molar ratio. We recently obtained high-affinity leptin antagonists using random mutagenesis followed by selection of high-affinity mutants by yeast surface display (Shpilman *et al.*, 2011). As a first step to using the same strategy to identify high-affinity PRL antagonists, we prepared del 1–9-G129R hPRL expressed on a yeast cell surface and showed that it retains its binding capacity for biotinylated hPRLR-ECD. These encouraging results suggest that such a methodology should be suitable for future development of high-affinity hPRL antagonist.

To prepare a long-lasting reagent for *in vivo* experiments, we developed a mono-pegylation protocol for hPRL that can also be applied to its del 1–9-G129R hPRL analogue. Interestingly, we found no difference between the affinity of the pegylated and non-pegylated hPRLs to hPRLR-ECD, indicating that pegylation does not interfere with site 1. On the other hand, the biological activity of the mono-pegylated analogue in both homologous (Baf3/LP) and heterologous (Nb2-11 C) *in vitro* bioassays was much lower than that of non-pegylated hPRL. This suggests that pegylation somehow interferes with hPRL's ability to induce the appropriate conformational changes within the pre-dimerized PRLR. As this step is assumed to rely on binding site 2, these data are consistent with the fact that the pegylation procedure that we used favors addition of PEG at the N terminus. Similar loss of activity has been previously observed with pegylated human growth hormone (Clark *et al.*, 1996) and leptin antagonists (Elinav *et al.*, 2009). However, the *in vivo* potency of the pegylated proteins was largely balanced by their prolonged half-life in the circulation.

In conclusion, we report procedures for large-scale production of various reagents that are widely used in the field of PRL research. In addition, we provide a rational basis for using yeast surface display as a screening method for random mutagenesis libraries aimed at improving receptor affinity of the PRLR antagonist del 1–9-G129R hPRL.

Acknowledgements

We thank the SPR unit at Weizmann Institute for the help in experiments.

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