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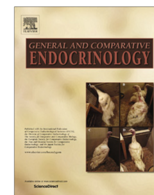

Comparison of *in vitro* bioactivity of chicken prolactin and mammalian lactogenic hormones

Ewa Ocłoń [a,b], Agnieszka Leśniak-Walentyn [a,b], Gili Solomon [a], Michal Shpilman [a], Anna Hrabia [b], Arieh Gertler [a,*]

[a] Robert H. Smith Faculty of Agriculture, Food and Environment, Hebrew University of Jerusalem, Rehovot 76100, Israel
[b] Department of Animal Physiology and Endocrinology, University of Agriculture in Krakow, Al Mickiewicza 24/28, 30-059 Krakow, Poland

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ABSTRACT

Recombinant chicken prolactin, expressed in *Escherichia coli* as an unfolded protein, was successfully refolded and purified to homogeneity as a monomeric protein. Its biological activity was evidenced by its ability to interact with rabbit prolactin receptor extracellular domain and stimulate prolactin receptor-mediated proliferation in three cell types possessing mammalian prolactin receptors. Chicken prolactin activity in those assays was 20–100-fold lower than that of mammalian lactogenic hormones, likely due to lower affinity for mammalian prolactin receptors and not to improper refolding, because in two homologous bioassays, chicken prolactin activity was equal to or higher than that of ovine prolactin and the CD spectra of chicken and human prolactin were almost identical. Our results using seven mammalian lactogenic hormones from five species in three bioassays revealed the major role of species specificity in testing biological activity *in vitro*. Heterologous bioassays may be misleading and homologous assays are strongly recommended for predicting the activity of species-specific lactogenic hormones *in vivo*.

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1. Introduction

Prolactin (PRL) is a polypeptide hormone that is mainly secreted by lactotroph cells of the anterior pituitary gland (Scanes et al., 1975; Freeman et al., 2000). Its amino acid sequence is similar to that of growth hormone, placental lactogen (PL) and the newly identified PRL-like protein that shares genomic, structural and biological features and belongs to the same protein family. The gene encoding avian PRL is located on chromosome 2 (Alipanah et al., 2011), and was initially described as containing five exons and four introns (Liu et al., 2008; Yousefi et al., 2012). The mature form of the protein contains 199 residues with three disulfide bridges between six cysteines, and has a molecular weight of ~23 kDa (Watahiki et al., 1989; Kansaku et al., 2008). However, little is known about the functions of PRL modification by phosphorylation, glycosylation, deamination, sulfonation and polymerization (Bédécarrats et al., 1999; Kansaku et al., 2005). Studies in birds have indicated that the PRL-encoding gene is expressed in the hypothalamus, pituitary, oviduct and ovary, as well as in the thymus, spleen and lymphocytes (Kansaku et al., 2008). This widespread expression partially explains its involvement in various processes, such as initiation and maintenance of incubation behavior, regulation of gonadal development and functions, egg-laying, osmoregulation and immunomodulation in poultry species (Harvey et al., 1984; El Halawani et al., 1986; Skwarło-Sońta, 1990; Ben-Jonathan et al., 1996; Li et al., 2011; Chaiseha and El Halawani, 2015). In view of prolactin pleotropic action preparation of recombinant chicken prolactin in amounts suitable for *in vivo* experiments as presented in our present work is timely. The diverse actions of PRL are mediated by the PRL receptor (PRLR), a single transmembrane protein that belongs to class I of the cytokine receptor superfamily, which includes growth hormone receptor, leptin receptor, interleukins, granulocyte colony-stimulating factor, granulocyte macrophage colony-stimulating factor, leukemia inhibiting factor, oncostatin M, erythropoietin, thrombopoietin, gp130 and ciliary neurotrophic factor (Xing et al., 2011). Several isoforms of membrane-bound PRLR have been identified: few variants of short, intermediate, long, and the soluble form containing only the extracellular domain (ECD) (Tanaka et al., 1992; Ohkubo et al., 1998; Clevenger and Kline, 2001). Upon binding to PRL, the PRLR can initiate multiple intracellular signaling cascades, including activation of the Janus kinase/signal transducers and

* Corresponding author at: Institute of Biochemistry, Food Science and Nutrition, Robert H. Smith Faculty of Agriculture, Food, and Environment, Hebrew University of Jerusalem, P.O. Box 12, Rehovot 76100, Israel.
E-mail address: arieh.gertler@mail.huji.ac.il (A. Gertler).

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activators of transcription (JAK2–STAT5), the mitogen-activated protein kinase (MAPK) and pAkt signaling pathways (Jiang et al., 2005).

To better characterize the biological properties of chicken PRL (chPRL) and to provide a valuable tool for pharmacological homology studies, a procedure for large-scale production of recombinant chPRL was established and the biological activity of the purified protein was tested *in vitro* in a homologous system. An additional question addressed in this paper was whether the various *in vitro* heterologous bioassays indeed reflect the homologous biological potential of lactogenic hormones *in vivo*. We tested seven mammalian lactogenic hormones along with chPRL in three heterologous *in vitro* bioassays in cells expressing human, rabbit or rat PRL receptors.

2. Materials and methods

2.1. Recombinant proteins, cells and chemicals

Recombinant human PRL (hPRL), rabbit and human PRL ECD (rbPRL-ECD and hPRL-ECD, respectively), ovine PRL (oPRL), rbPRL, rat prolactin (rPRL), human placental lactogen (hPL), oPL and bovine placental lactogen (bPL) were prepared in our laboratory as described previously (Gertler et al., 1992, 1996, 1998; Sakal et al., 1997; Leibovich et al., 2001). Streptavidin-horseradish peroxidase (Streptavidin – HRP) was purchased from Jackson ImmunoResearch (West Grove, PA, USA). Baf3 cells stably transfected with the long form of rbPRLR were from Dr. Jean Djiane, Nb2-11C rat lymphoma cell line was from Dr. Henri Friesen, and Baf3/LP cells stably transfected with the long form of hPRLR were from Dr. Vincent Goffin. *Escherichia coli* bacterial strain A 1645 expressing chPRL was a gift from Biotechnology General Israel Inc. (Beer Tuvia, Israel). This strain constitutively produces thermostable gamma repressor at 30 °C which prevents transcription (Roberts et al., 1968). When the temperature is raised to 42 °C, repression is abolished. All other reagents were of analytical grade.

2.2. Expression, refolding, and purification of chPRL

E. coli cells (500 ml) were grown in a 2.5-L flask in LB medium (1% bacto-tryptone, 0.5% yeast extract, 1.5% NaCl, w/v) at 30 °C to an optical density at 600 nm (OD_{600}) of 1.0 and temperature was then raised to 42 °C. The cells were grown for an additional 4 h, pelleted for 6 min at 6000*g*, and frozen. The bacterial pellet from 5 L of culture was thawed on ice and resuspended in lysis buffer (10 mM Tris-HCl, 10 mM EDTA, pH 8) containing 0.5 mg lysozyme/ml. Inclusion bodies (IBs) were then prepared as described previously and frozen (Gertler et al., 1998). IBs obtained from 5 L of bacterial culture were then solubilized in 50 ml of 50 mM Tris base, pH 8.5, containing 6 M guanidine-HCl and 8 mM 1.4-dithiothreitol (DTT). After 45 min of stirring at room temperature, the solution was slowly added by peristaltic pump to 1000 ml refolding buffer (50 mM Tris base, 160 mM arginine, 1 M urea, 4 mM cysteine, pH 8.5), stirred at 4 °C for 24 h, and then the clear solution was dialyzed against 3 × 10 L of 10 mM Tris-HCl, pH 8 at 4 °C. The resultant solution was applied to a Q-Sepharose column (2.5 × 6 cm) pre-equilibrated with 10 mM Tris-HCl, pH 8. Elution was carried out using a discontinuous NaCl gradient in the same buffer (50, 100, 150, 300 mM NaCl). Fractions (40 ml) were collected and protein concentration was determined at OD_{280}. Fractions containing the monomeric chPRL were identified using size-exclusion chromatography (SEC) on an analytical Superdex 75 HR column in TN buffer (25 mM Tris-HCl, 300 mM NaCl, pH 8) at room temperature. Fractions containing the monomeric chPRL were pooled (tubes 23–32 in Fig. 2), dialyzed against $NaHCO_3$ to ensure a 4:1 protein-to-salt ratio and lyophilized.

2.3. Determination of purity and monomer content

SDS-PAGE was carried out according to Laemmli (1970) in a 10% polyacrylamide gel under reducing and non-reducing conditions. The gel was stained with Coomassie Brilliant Blue R. Gel-filtration chromatography was performed on a Superdex 75 HR 10/30 column with 0.2-ml aliquots of the Q-Sepharose column-eluted fraction using TN buffer.

2.4. Circular dicroism spectra (CD)

The CD spectra in millidegrees were measured with an AVIV model 62A DS circular dichroism Spectrometer (Aviv Assoc., Lakewood, NJ) using a 0.020 cm rectangular QS Hellma cuvette. The spectrometer was calibrated with camphorsulfonic acid. The absorption spectra were measured with an AVIV model 17DS UV–vis-IR spectrophotometer (Aviv Assoc., Lakewood, NJ) using a 1.000 cm QS cuvette and corrected for light-scattering. Lyophilized chicken and human prolactins were dissolved in water, pH 8.0, and adjusted to 25 μM concentrations. The CD spectra were recorded by using a J-810 spectropolarimeter (JASCO) equipped with a Peltier thermostat using the supplied Spectra Manager software in a 0.1 cm quartz cuvette in the proteins storage buffer. Far-UV CD spectra were collected over 190–260 nm at 25 °C. For the secondary-structure determination, the CD data were expressed in degree $cm^2\ dmol^{-1}$ per mean residue, based on a respective molecular mass.

2.5. Binding assay

In-house-prepared biotinylated hPRL served as the ligand in the competitive binding experiment, chPRL and hPRL as competitors and rbPRLR-ECD as the receptor source. Polystyrene microtiter plates (96-well) were coated overnight at 4 °C with 100 μl of rbPRLR-ECD (0.8 μg/ml) in phosphate buffered saline (PBS) pH 7.4. Wells were then washed once with PBST (PBS containing 0.05% w/v Tween 20) and blocked with PBS containing 3% (w/v) skim milk for 2 h at room temperature. Wells were washed again with PBST and incubated with different concentrations of unlabeled chicken and human PRLs (50 μl/well, in triplicate) for 30 min, and then 50 μl of biotinylated hPRL (0.85 μg/ml) was added to each well for another 1.5 h. Then the wells were washed three times with PBST and incubated with 1:5000 streptavidin-HRP in PBST for 1 h. The plate was again washed three times with PBST, then developed using 100 μl/well 3,3′,5,5′-tetramethylbenzi dine (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, USA).

2.6. Biological activity in vitro in the Nb2-11C, Baf3/rbPRLR and Baf3/hPRLR heterologous bioassays

Cell line Nb2-11C expressing the short form of rPRLR was grown as a suspension culture in 75-cm^2 tissue-culture flasks (Nunc, Kamstrup, Roskilde, Denmark). For maximal growth and routine maintenance, cells were cultured in RPMI-1640 medium containing 5% (v/v) fetal calf serum (FCS) supplemented with antibiotic–antimycotic solution (10,000 units penicillin, 10 mg streptomycin and 25 μg amphotericin B per ml). The cells were incubated under a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. Stationary cultures were obtained by transferring the Nb2 cells into lactogen-free medium in which FCS was replaced with 5% (v/v) horse (gielded) serum. The experiment was performed in 96-well plates seeded with 2.5×10^4 cell/well.

Baf3/rbPRLR cells (stably transfected with rbPRLR) were grown as suspension cultures in 75-cm^2 tissue-culture flasks. For maximal

growth and routine maintenance, cells were cultured in RPMI-1640 medium containing 5% FCS supplemented with antibiotic–antimycotic solution and 0.5 ng/ml recombinant oPRL. Baf3/LP/hPRLR cells stably transfected with hPRLR were grown and treated similarly to Baf3/rbPRLR cells except that oPRL was replaced with hPRL.

Prior to the experiment, the cells were washed three times with RPMI-1640 medium containing 5% horse serum, suspended in the same medium at 2.5×10^4 cell/ml and seeded in the 96-well plate as already described. In all assays, hormone-dependent cell proliferation was determined by 3-(4,5-dimethylthiazolyl-2)-2,5-diphe nyltetrazolium bromide (MTT) assay 48 h after hormone addition. Growth curves were drawn using the Prizm (4.0) (Prisma, GraphPad Prism version 4.0, GraphPAD Software, San Diego, CA, USA) nonlinear regression sigmoidal dose response curve and the EC_{50} values were calculated.

2.7. Biological activity in homologous assays

The experiment was carried out with Hy-Line laying hens (n = 6) at the age of 23 weeks, caged individually under a photoperiod of 14L:10D with free access to food and water as described previously by Hrabia et al. (2004). Briefly, birds were decapitated 22 h before expected time of ovulation. The following prehierarchical follicles were isolated from the ovary: small white (1–4 mm in diameter), large white (4–6 mm) and yellowish (6–8 mm). Whole follicles were randomly assigned to 1 ml of Eagle's medium containing recombinant chicken or ovine PRL at a dose of 0 (control), 78.1, 312.5 or 1250 ng/ml, 0.05 g/ml bovine serum albumin (BSA) and 2 μl/ml antibiotic–antimycotic solution. From each ovary, either six small white follicles pooled together, 1 large white follicle or 1 yellowish follicle were incubated in a 24-well multidish at 38 °C for 24 h at each PRL dose. After incubation, the medium was collected. The secretion of estradiol was measured radioimmunologically with a commercial kit (DIA source ImmunoAssays S.A., Louvain-la-Neuve, Belgium) and was expressed per follicle. The detection limit was 6.4 pg/ml, recovery was 90.05% and cross-reactivity of estradiol antiserum for estradiol, estron, and estriol was 100%, 1.0%, and 0.6%, respectively. The intra- and interassay coefficients of variation were 6.1% and 12.15%, respectively.

To test the effect of PRLs on leukocyte proliferation, blood samples were collected into heparinized test tubes from the wing vein of chickens (n = 6) and mixed 1:1 with RPMI 1640 medium containing L-glutamine and supplemented with penicillin (100 unit/ml) and streptomycin (100 μg/ml). The cell suspensions were then layered onto histopaque 10774 and centrifuged at 220*g* for 30 min to separate out the leukocytes. The white blood cell layer was removed and washed twice. Leukocytes were adjusted to 1×10^7 viable cell/ml in RPMI 1640 with 10% (v/v) heat-inactivated fetal bovine serum (FBS). Using trypan blue exclusion, cell viability was determined to be ⩾95%. Leukocytes were then plated in triplicate at a density of 5×10^5 per well in multidish 96-well plates in the presence of serial dilutions of recombinant chicken or ovine PRL (0.076, 0.31, 1.20, 4.88, 19.53, 78.13, 312.50, 1250, 5000 ng/ml). Control cultures consisted of the cells incubated with supplemented culture medium alone. The cultures were incubated for 48 h under a humidified 5% CO_2/95% air atmosphere at 42 °C. The proliferation of leukocytes was determined by MTT assay 48 h after hormone addition.

3. Results

3.1. Purification and chemical characterization of recombinant chPRL

Induction of *E. coli* cells (4 clones) by temperature shift from 30 to 42 °C resulted in a main band of ~23 kDa, corresponding to chPRL, in the IBs (not shown) and one of the clones was chosen for large-scale purification. Chicken PRL was then expressed in 5 L bacterial culture refolded and purified to homogeneity on a Q-Sepharose anion-exchange column equilibrated with 10 mM Tris-HCl buffer, pH 8, using a non-continuous NaCl gradient (Fig. 1). The fractions eluted by 50 and 100 mM NaCl (Fig. 1, tubes 17–33) contained over 95% monomeric PRL and 5% dimers and were over 95% pure, as detected by SDS-PAGE under reducing and non-reducing conditions, (Fig. 2, left). In parallel SEC analysis (Fig. 2, right) showed that it is over 90% monomer. Fractions eluted with higher NaCl concentrations contained higher amounts of dimers and oligomers. Fractions containing the monomeric protein were pooled, concentrated by ultrafiltration, dialyzed and lyophilized as described in Section 2. The specific absorbance at 280 nm, calculated by the DNAMAN program, was 1.151 for a 0.1% protein solution. Six similar preparations were carried out and the yield of the monomeric fraction varied from 44 to 56 mg from 5 L of bacterial culture. The lyophilized chPRL was stored at −20 °C and was fully stable for at least 8 months, as determined by SDS-PAGE and SEC profile, as well as by biological activity (not shown). CD spectra analysis (Fig. 3) indicates that chPRL is almost identical to hPRL and its spectrum is quite characteristic for cytokines having the 4-bundle of α-helical structure.

3.2. SEC analysis of complexes of rbPRLR-ECD with chPRL and hPRL on Superdex 75 column

To characterize the binding stoichiometry between chPRL and rbPRLR-ECD, the respective complex components were mixed in several molar ratios; this showed that the complex consisted of a 1:1 ratio, as shown previously for several mammalian PRLs (Bignon et al., 1994; Gertler et al., 1996; Sakal et al., 1997; Leibovich et al., 2001). To compare the stability of the 1:1 chPRL–rbPRLR-ECD complex to that of hPRL–rbPRLR-ECD at a 1:1 M ratio, we employed SEC on an analytical Superdex 75 column, which determines the molecular mass of the complex under non-denaturing conditions. The experiments were performed using a constant 8 μM of the respective ligand and 8 μM of rbPRLR-ECD. The stoichiometry was evidenced by the appearance of a single main peak for the complex with retention time (RT) varying from 16.01 to 16.05 min, whereas the RT of purified chPRL was 17.27 min (Fig. 2) and that of rbPRLR-ECD was 17.31 min (not shown). The molecular mass calculation, based on the corresponding peak RT, was approximately 45 kDa, indicating 1:1 stoichiometry.

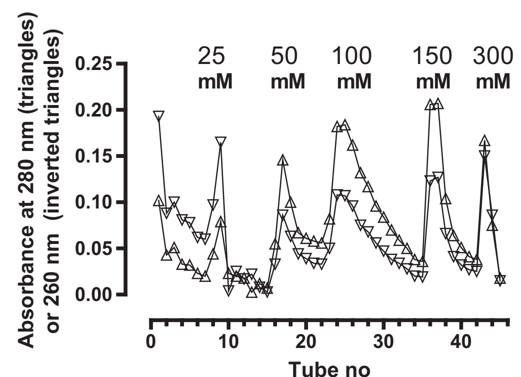

Fig. 1. Separation of recombinant chPRL on DEAE cellulose column. The column (2.5 × 6 cm) was equilibrated with 10 mM Tris-HCl, pH 8.0, at 4 °C. The dialyzed solution of refolded protein was applied to the column at a rate of 120 ml/h. Elution was carried out using a discontinuous NaCl gradient in the same buffer at 120 ml/h, and 40-ml fractions were collected. The numbers above each peak indicate the concentration of NaCl in the elution buffer. Protein concentration was determined by absorbance at 280 and 260 nm. Every peak was assayed for chPRL content profile by SEC in a Superdex™ 75 HR column. Tubes, eluted with 50 and 100 mM NaCl contained monomeric protein and were pooled.

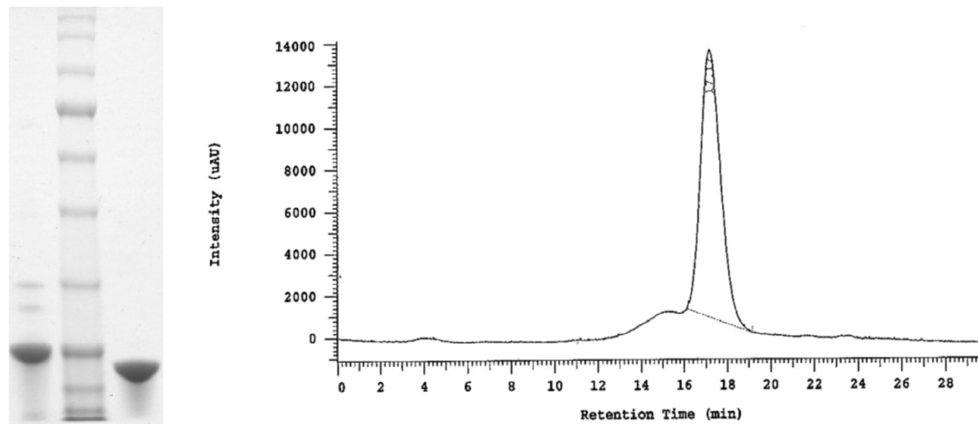

Fig. 2. Left figure – SDS-PAGE (10%) of purified chicken prolactin (5 μg) – left line in presence and right lane absence of reducing agent. The molecular mass of the main band run in the presence of reducing agent is ~25 kDa, whereas the molecular mass of the main band run in an absence of reducing agent is ~23 kDa, close to the theoretical calculated mass of 22,735 Da. It is well documented that in the absence of reducing agent globular proteins run faster on the SDS-PAGE gels. Middle lane – molecular mass markers from the bottom to the top in kDa: 10, 15, 20, 25 (darker), 35, 50, 75, 100 (darker), 150, 200 and 240. Right figure – SEC analysis of purified chicken prolactin (100 μg) on G-75 Superdex column developed with TN buffer pH 8.0. For other details see text.

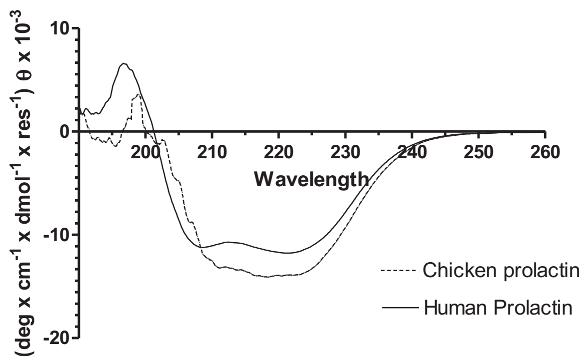

Fig. 3. CD spectra of purified recombinant chicken and human prolactins at 25 μM concentration. For other details see text.

Gradual dilution of the respective 1:1 complexes of hPRL and rbPRLR-ECD from 8 μM to 0.125 μM changed the RT of the complex peak only slightly, from 16.01 to 16.10 min. In contrast, similar dilution of the 1:1 chPRL–rbPRLR-ECD complex resulted in a gradual shift from RT of 16.05 min for the complex at 8 μM to 16.18, 16.43, 17.07, 17.28 and 17.48 min for the complexes diluted, respectively, to 4, 2, 0.5, 0.25 and 0.125 μM, indicating that the complex was gradually dissociated to its components. As noted, retention time (RT) of ~17.27 min is characteristic for pure chPRL (Fig. 3) and that of rbPRLR-ECD is 17.3 min (not shown).

3.3. Comparison of biological activity of chPRL and several mammalian PRLs and PLs using heterologous bioassays in three mammalian cell lines

The biological activities of chPRL were compared to those of several mammalian PRLs (hPRL, oPRL, rbPRL, rPRL) and PLs (hPL, oPL, bPL) in three cell lines, using a bioassay based on proliferation: (a) Baf3 cells stably transfected with rbPRLR, (b) Baf3/LP cells stably transfected with hPRLR, and (c) NB2-11C cells expressing the short form of rPRLR. All three cell lines were incubated with increasing concentrations of various mammalian PRLs or PLs over a final range of 0.076–5000 ng/ml for a period of 48 h, and viable cells were quantified by MTT assay (Fig. 4A–C). The potencies of the individual lactogenic hormones were calculated and are presented as EC_{50} values in Table 1. The most potent ligand for cells transfected with rbPRLR was hPRL. The potency of chPRL was approximately 18-fold lower, whereas rbPRL (the homologous ligand) and hPL were 6-fold less potent (Table 1). Ovine PRL and bPL were stronger agonists, whereas rPRL and oPL were both approximately 4-fold less potent (Table 1).

To analyze and compare the bioactivities of chPRL and hPRL with PRLs from multiple mammalian subprimate species, we also used the Baf3/LP cell line stably transfected with human PRLR (Table 1). As expected, hPRL was the most potent ligand, whereas chPRL was only a weak agonist, with an EC_{50} value that was 114-fold higher. The potency of hPL was similar to that of chPRL as evidenced by comparable EC_{50} values (Table 1). Compared to hPRL, rPRL, oPRL, oPL and bPL were, respectively, 78-, 13- and ~5-fold less potent. Surprisingly, rbPRL was not active at all (Table 1). To verify that chPRL was biologically active, the same panel of PRLR ligands was also tested in the highly sensitive and broadly used rat Nb2-11C proliferation bioassay for lactogenic activity. In this bioassay, chPRL had the lowest bioactivity (Table 1), and bioactivities of oPRL and oPL were comparable. Bovine PL had the highest activity, whereas rbPRL and hPL had comparable EC_{50} values and were about 20-fold less potent than hPRL (Table 1).

3.4. Binding assay

The binding activity of chPRL was compared to that of hPRL in a competitive non-radioactive binding assay using biotinylated hPRL as the ligand and rbPRLR-ECD as the receptor. Both chicken and human PRLs displaced the biotinylated hPRL with respective IC_{50} values of 2.9×10^{-5} M and 1.5×10^{-7} M, indicating a ~200-fold difference in affinity toward rbPRL-ECD (Fig. 4D). The difference in affinity corresponded to the results of the SEC analysis, demonstrating in a semi-quantitative way the lower affinity of chPRL.

3.5. Effects of chicken and ovine PRLs in homologous bioassays

To determine whether recombinant chPRL is also active in homologous bioassays, we tested its effects on *in vitro* estradiol secretion by whole prehierarchical ovarian follicles of chicken and on chicken leukocyte proliferation in comparison to mammalian (ovine) PRL. As shown in Table 2, chPRL added to the incubation medium at doses of 78.1 ng/ml, 312.5 ng/ml and 1250 ng/ml significantly decreased estradiol secretion by the small white follicles by 46.4%, 56.7% and 71.8%, large white follicles by 58.3%,

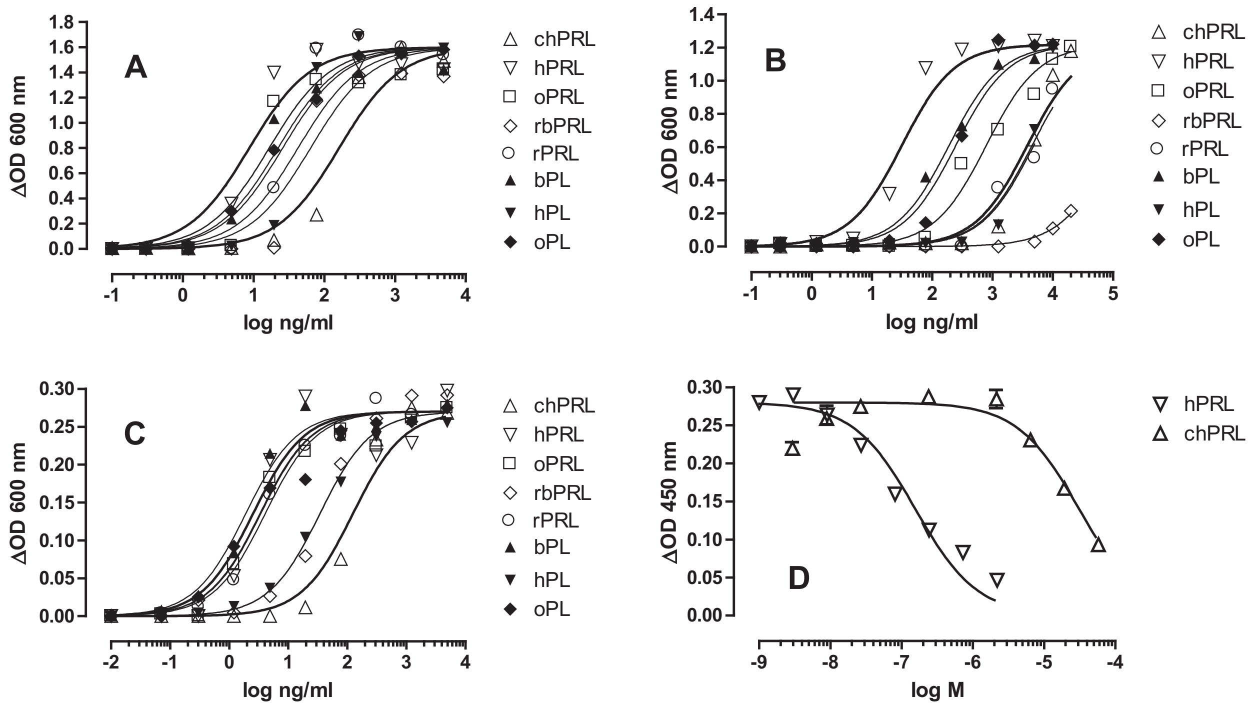

Fig. 4. Comparison of biological activity of chicken prolactin to several mammalian prolactins or placental lactogens using bioassays in 3 mammalian cell lines and binding assay. Graphs representing the response to human and chicken prolactins are shown in bold lines. (A) Baf3 cells stably transfected with rabbit PRLR; (B) Baf3/LP cells stably transfected with human PRLR; (C) Nb2-11C rat lymphoma cells. (D) Binding assay comparing human and chicken PRLs (see text).

Table 1
Comparison of biological activity of chicken prolactin to several mammalian lproprolactins and placental lactogens using bioassays in three mammalian cell lines.

Hormone tested	EC_{50} value for each cell line (ng/ml)[a]		
	Baf3/rbPRLR[b]	Baf3/LP/hPRLR[c]	Rat Nb2-11C[d]
Chicken prolactin	138 (115–161)	4235 (3765–4706)	327 (135–523)
Human prolactin	7.46 (7.38–7.55)	37.1 (31.1–43.6)	2.26 (2.05–2.48)
Ovine prolactin	11.6 (9.7–13.6)	483 (478–488)	2.70 (2.40–3.08)
Rabbit prolactin	50.2 (45.9–54.5)	Nil	45.8 (38.1–53.6)
Rat prolactin	28.3 (25.5–31.1)	2835 (1773–3898)	4.99 (4.39–5.60)
Human placental lactogen	50.5 (38.6–62.4)	5327 (4972–5683)	43.6 (34.8–52.4)
Ovine placental lactogen	31.8 (22.8–40.6)	205 (193–298)	2.73 (2.58–2.88)
Bovine placental lactogen	16.5 (16.2–16.6)	168 (143–194)	1.49 (1.12–1.87)

[a] EC_{50} value is given in ng/ml of the respective protein in the tested solution; 10 µl was added to 100 µl of the relevant cell suspension. The values in parentheses show the range obtained from two to three experiments.
[b] Cell line stably transfected with rabbit prolactin receptor (rbPRLR) (Dusanter-Fourt et al., 1994).
[c] Cell line stably transfected with human prolactin receptor (hPRLR) (Bernichtein et al., 2003).
[d] Cell line expressing the short form of rat prolactin receptor (Gertler et al., 1985).

Table 2
Effect of chicken and ovine prolactins on *in vitro* estradiol secretion (pg/follicle per 24 h) by chicken whole prehierarchical ovarian follicles.

Follicle	Prolactin (ng/ml)	0	78.1	312.5	1250
Small white[a]	Chicken	8.85 ± 0.49	4.75 ± 0.49[***]	3.83 ± 0.45[***]	2.50 ± 0.45[***]
(1–4 mm)	Ovine	7.71 ± 0.45	6.34 ± 0.38	4.47 ± 0.35[***]	5.67 ± 0.53[*]
Large white[a]	Chicken	15.8 ± 0.99	6.58 ± 0.50[***]	5.73 ± 0.53[***]	4.80 ± 0.55[***]
(4–6 mm)	Ovine	16.3 ± 0.45	15.4 ± 0.61	7.05 ± 0.51[***]	8.13 ± 0.72[**]
Yellowish[a]	Chicken	9.93 ± 0.59	7.21 ± 0.83[*]	6.38 ± 0.60[***]	7.37 ± 0.41[**]
(6–8 mm)	Ovine	9.13 ± 0.53	5.90 ± 0.66[**]	6.49 ± 0.68[*]	5.81 ± 0.34[***]

[a] Each value represents the mean ± SEM of six determinations.
[*] $P < 0.05$.
[**] $P < 0.01$.
[***] $P < 0.001$ relative to control, as determined by Student's *t*-test.

63.7% and 69.6%, and yellowish follicles by 27.4%, 35.7% and 25.7%, respectively. Ovine PRL had a similar effect on yellowish follicles, whereas its effect was less pronounced in white follicles. In the leukocyte experiment (Table 3), both chicken and ovine PRLs similarly and significantly increased the proliferation of chicken leukocytes.

Table 3
Effect of chicken prolactin (chPRL) and ovine prolactin (oPRL) on chicken peripheral blood leukocyte proliferation.

ng/ml	0	0.076	0.305	1.2	4.89	19.5	78.1	312.5	1250	5000
chPRL[a]	0.125 ± 0.004	0.293 ± 0.013*	0.322 ± 0.014*	0.334 ± 0.016*	0.329 ± 0.015*	0.365 ± 0.018*	0.345 ± 0.016*	0.334 ± 0.015*	0.383 ± 0.016*	0.404 ± 0.022**
oPRL[a]	0.130 ± 0.007	0.337 ± 0.021*	0.348 ± 0.014*	0.347 ± 0.018*	0.356 ± 0.015*	0.389 ± 0.016*	0.383 ± 0.016*	0.387 ± 0.016*	0.386 ± 0.015*	0.405 ± 0.017**

[a] Each value represents the mean ± SEM of three determinations.
* $P < 0.05$.
** $P < 0.01$ relative to control, as determined by Student's *t*-test.

4. Discussion

Using a relatively simply protocol, we produced tens of milligrams of pure recombinant chPRL from 5 L of bacterial culture, thus providing a research tool for future investigations on both *in vitro* and *in vivo* scales. The protein was pure as judged by SDS-PAGE, and over 95% monomeric. Determination of its lactogenic activity included determining its ability to interact with rbPRL-ECD in SEC and binding assays, PRLR-mediated proliferation assays in three cell lines possessing mammalian PRLRs, and two *in vitro* homologous bioassays. The heterologous bioassays clearly showed that though chPRL has the capacity to interact with rbPRLR-ECD, forming a 1:1 complex like other mammalian lactogenic hormones (Bignon et al., 1994; Gertler et al., 1996; Sakal et al., 1997; Leibovich et al., 2001), its affinity toward for this soluble receptor is much lower than that of hPRL. This lower affinity is likely the reason for the low biological activity in cells possessing mammalian PRLRs (Table 1 and Fig. 4), which was also one to two orders of magnitude lower than that of hPRL. This finding raised the question of whether chPRL's lower affinity results from improper refolding or is an intrinsic property. To answer this, we compared the CD spectra of chicken and human prolactins and found them very similar and performed two *in vitro* experiments in a homologous model, comparing the effects of chPRL and oPRL on *in vitro* estradiol secretion by whole chicken prehierarchical ovarian follicles and proliferation of chicken peripheral blood leukocytes. The results confirmed those from previous experiments, in which oPRL decreased *in vitro* secretion of estradiol by chicken white ovarian follicles by 39–66% (Hrabia et al., 2004), and in which oPRL suppressed LH-stimulated estradiol secretion by very small ovarian follicles (Zadworny et al., 1989). With respect to the effect of the examined PRLs on leukocyte proliferation, our results were in agreement with those of Skwarło-Sońta (1990), who revealed a direct, stimulatory action of bPRL on White Leghorn cockerel lymphocyte proliferation. Whereas in heterologous bioassays, the activity ratio of oPRL to chPRL varied from 12- to 121-fold, in the homologous assay, chPRL activity was either equal to or higher than that of oPRL, strongly indicating correct refolding and the importance of species specificity in *in vitro* biological activity. This conclusion is also supported by the CD analyses in which chPRL were compared to hPRL. The finding that in homologous bioassays, chPRL is at least as active as oPRL raised the question of why its activity was so much lower in the heterologous assays. To answer this, we compared the primary structures of chPRL to those of human and ovine PRLs (Table 4). The vast majority of the 35 residues found important for the binding of hPRL to hPRLR—by analysis of the complex's crystal structure (Svensson et al., 2008) or by site-directed mutagenesis (Luck et al., 1991) (enlarged bold letters in Table 4) also exist in chPRL. Only five amino acids (G16, Y31, V55, V188 and S197) are different. Future mutagenesis of these residues to those existing in human or ovine PRLs will thus explain the reason for the low activity in heterologous assays.

To further study the importance of species specificity in evaluating PRLs' and other lactogenic hormones' activity *in vitro*, we employed three bioassays in cells possessing human, rabbit and rat (the short form) PRLR, and used seven recombinant lactogenic hormones from five mammalian (human, rat, rabbit, ovine, bovine) species. The results clearly showed that species specificity plays a very important role in determining PRL activity *in vitro*. Even in the NB2-11C bioassay, the most widely used *in vitro* test of lactogenic hormone activity the activities of hPRL, oPRL, oPL and bPL were in a similar range, the activity of rPRL was ~2-fold lower and the activities of hPL and rbPRL were ~20-fold lower. Using another assay in cells stably transfected with rbPRLR, the differences between hPL and rbPRL and hPRL, oPRL, oPL and bPL were much

Table 4
Sequence alignment of chicken, human and ovine prolactins. Residues highlighted in yellow contribute to binding to of human prolactin to human prolactin receptor according to Luck et al. (1991), residues highlighted in gray contribute to such binding according to Svensson et al. (2008) and residues indicated by both papers are highlighted in light blue. The residues in chicken prolactin that differ from the highlighted residues in human and ovine prolactins are underlined and marked in red letters.

chPRL	1	LPICPIGSVN	CQVSLGELFD	RAVKLSHYIH	YLSSEIFNEF	DERYAQGRGF	50
hPRL	1	LPICPGGAAR	CQVTLRDLFD	RAVVLSHYIH	NLSSEMFSEF	DKRYTHGRGF	50
oPRL	1	TPVCPNGPGN	CQVSLRDLFD	RAVMVSHYIH	NLSSEMFNEF	DKRYAQGKGF	50
chPRL	51	ITKAVNGCHT	SSLTTPEDKE	QAQQIHHEDL	LNLVVGVLRS	WNDPLIHLAS	100
hPRL	51	ITKAINSCHT	SSLATPEDKE	QAQQMNQKDF	LSLIVSILRS	WNEPLYHLVT	100
oPRL	51	ITMALNSCHT	SSLPTPEDKE	–AQQTHHEVL	MSLILGLLRS	WNDPLYHLVT	100
chPRL	101	EVQRIKEAPD	TILWKAVEIE	EQNKRLLEGM	EKIVGRVHSG	DAGNEIYSHW	150
hPRL	101	EVRGMQEAPE	AILSKAVEIE	EQTKRLLEGM	ELIVSQVHPE	TKENEIYPVW	150
oPRL	101	EVRGMKGVPD	AILSRAIEIE	EENKRLLEGM	EMIFGQVIPG	AKETEPYPVW	150
chPRL	151	DGLPSLQLAD	EDSRLFAFYN	LLHCLRRDSH	KIDNYLKVLK	CRLIHDSNC	199
hPRL	151	SGLPSLQMAD	EESRLSAYYN	LLHCLRRDSH	KIDNYLKLLK	CRIIHNNNC	199
oPRL	151	SGLPSLQTKD	EDARHSAFYN	LLHCLRRDSS	KIDTYLKLLN	CRIIYNNNC	199

less pronounced. In contrast, in cells stably transfected with hPRLR, the differences between the EC_{50} values for hPRL and the other hormones were one or even two orders of magnitude higher and in the case of rbPRL, no activity was observed at all. Obviously, the number of receptors on the cell surface affects the bioassay's sensitivity, but probably not species specificity. We therefore conclude that there is no one single optimal *in vitro* bioassay for all lactogenic hormones; the heterologous bioassays may be misleading, and only the homologous assays are valid for measuring prolactin activity. Therefore, our present work emphasizes the need to produce large amounts of recombinant homologous chPRL to conduct *in vivo* experiments aimed at elucidating regulation of growth, metabolism and reproduction.

Declaration of interest

No conflicts of interest, financial or otherwise, are declared by the authors.

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Author contribution

EO, AH and AG planned the experiments and wrote the paper, EO, ALW, GS, MS and AH conducted the experiments.

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