Isolation of cleaved prolactin variants that stimulate DNA synthesis in specific cell types in rat pituitary cell aggregates in culture

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Using an affinity column of monoclonal antibodies against rat prolactin (PRL), PRL immunoreactive material secreted by rat pituitary aggregate cell cultures was purified in milligram amounts from the culture medium. Further purification of the PRL immunoreactive molecules was achieved by anion-exchange and reversed-phase h.p.l.c. At least four variants could be distinguished which showed PRL-like bioactivity in the Nb2 lymphoma bioassay. On SDS/PAGE two variants migrated as single bands under both reducing and non-reducing conditions. The two other variants migrated as single bands under non-reducing conditions, but yielded under reducing conditions two fragments, the larger of which had molecular masses of approx. 16 and 17 kDa respectively. These variants were therefore considered to be cleaved PRL (clPRL) variants, and were designated clPRL-1 and clPRL-2 respectively. These variants were not artefactually produced at low pH during the h.p.l.c. purification step. Each of the clPRLs represented about 0.6–1.0 % of total PRL. The cleavage site of clPRL-1, determined by N-terminal and C-terminal amino acid sequence analysis, was in the large disulphide loop between Tyr-145 and Leu-146. Purified clPRL-1 added to pituitary aggregate cell cultures from 14-day-old female rats in the presence of the dopamine agonist bromocriptine increased [³H]thymidine incorporation into DNA in gonadotrophs and thyrotrophs, but not in other pituitary cell types. Under the same experimental conditions the main forms of PRL and clPRL-2 were inactive. These data demonstrate that PRL is cleaved within the pituitary gland between specific amino acid residues, and that PRL cleaved between Tyr-145 and Leu-146 may have a specific biological role as a paracrine growth regulator in this tissue.

INTRODUCTION

Prolactin (PRL) is a polypeptide hormone secreted by the anterior pituitary gland. It is a single polypeptide chain with three disulphide loops and a molecular mass of 24 kDa. However, small amounts of charge variants [1–5], polymers [5,6], glycosylated [5,7] and cleaved forms [8] have been reported. Because of the diversity in physiological functions of PRL, this molecular heterogeneity has raised the question of the existence of PRL variants with a specific function [9–11].

Mitra [8] isolated a form of PRL cleaved in the large disulphide loop, which yielded under reducing conditions two fragments of respectively 16 and 8 kDa. This cleaved PRL (clPRL) is synthesized and secreted by the anterior pituitary of rats [8], mice [12] and humans [13], and is also formed by cleavage of PRL in target tissues such as the mammary gland [14] and the prostate [14,15].

Several groups have tried to find a physiological function for clPRL in target tissues. Mitra [16] purified the 16 kDa fragment of clPRL extracted from the pituitary and showed that it had mitogenic activity on mammary epithelial cells *in vivo*, whereas intact PRL was ineffective. In a later study, however, Clapp *et al.* [17], using clPRL and the 16 kDa fragment formed from PRL by a subcellular fraction of the rat mammary gland, found that intact PRL and clPRL had the same mitogenic potencies in mammary tissue, whereas the 16 kDa fragment was less potent. In a standard radioreceptor assay using membrane fractions of liver or prostate, no difference in binding between intact PRL and clPRL was seen [14]. Another study revealed that the 16 kDa fragment binds to PRL receptors with an activity that varies

between the target tissues, and that specific receptors for the 16 kDa fragment may exist [18]. Target tissues also differ in their PRL-cleaving potencies according to different physiological states [19]. However, some investigators doubt that the cleavage of PRL is a physiological phenomenon, and even suggest it is an artifact of the extraction procedure [20].

To our knowledge, the question of a possible role of clPRL within the anterior pituitary has not been raised. This question is reasonable, since clPRL is produced in much smaller amounts than the main 24 kDa PRL molecule secreted as a hormone in the general circulation [8,12,13]. The fact that such small amounts are produced may suggest a local paracrine action, since very small amounts of clPRL would be sufficient for eliciting local effects. Another important open question concerns the cleavage site of clPRL formed in the anterior pituitary.

In the present investigation we isolated two forms of clPRL, secreted by anterior pituitary cell cultures, as their native two-polypeptide chain structure using an affinity column of monoclonal antibodies against rat PRL, ion-exchange chromatography and reversed-phase h.p.l.c. Both clPRLs were tested for PRL-like mitogenic activity in the Nb2 bioassay. It is well known that PRL and PRL-like molecules share the characteristics of promoting growth and differentiation and of being mitogenic in a variety of tissues [16,17,21–23]. The plasma membrane receptor of PRL (as well as of growth hormone) belongs to the cytokine receptor superfamily [24], the ligands of which are all involved in differentiation and growth of disparate cell types. We therefore searched for a possible mitogenic function of clPRLs on certain anterior pituitary cell types. The cleavage site of one clPRL could be determined.

Abbreviations used: (r)PRL, (rat) prolactin; PRL-ir, PRL immunoreactivity; clPRL, cleaved prolactin; PBS, phosphate-buffered saline; SFM, serum-free Fisher's medium; TFA, trifluoroacetic acid.

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A preliminary report on some of these findings has been presented in abstract form [25].

MATERIALS AND METHODS

Animals

For the production of monoclonal antibodies, 6–10-week old female Balb/c mice were used. Rats used in this study were 14-day-old female, adult male or female (random cycle) Wistar rats. All animals were kept in an environment of constant temperature, humidity and day–night cycle, and received pellet food and water *ad libitum*. Mice were killed by spinal dislocation, rats by decapitation.

Reaggregated rat anterior pituitary cell cultures and collection of conditioned culture medium

Conditioned medium was obtained by pooling spent culture medium of anterior pituitary reaggregate cell cultures used for various experimental purposes in our laboratory.

Anterior pituitary cells were enzymically dispersed into single cells as described previously [26,27]. Three-dimensional cell aggregates (reaggregate cell cultures) were formed by continuous gyratory shaking in untreated plastic Petri dishes (35 mm diam.; Nunc, Roskilde, Denmark) [(1–2) × 10⁶ cells/2 ml of culture medium] [26,27]. The culture medium was a serum-free medium consisting of Dulbecco's modified Eagle's medium and Ham's F-12 nutrient mixture (1:1, v/v; Gibco, Renfrewshire, Scotland, U.K.), 15 mM-Hepes buffer, 15 mM-Tes buffer, 20 µM-ethanolamine, 20 nM-sodium selenite, 10 mM-ethanol, NaHCO₃ (1 g/l), transferrin (40 mg/l), insulin (5 mg/l), catalase (1 mg/l), BSA (5 g/l), penicillin (35 mg/l) and streptomycin (50 mg/l), with or without addition of Phenol Red (0.8 mg/l), dexamethasone (4 or 80 nM), tri-iodothyronine (0.05–5 nM) and/or 1 nM-oestradiol. After 2 days, the medium was renewed and the aggregated cells were transferred to untreated Petri dishes (50 mm diam.; 4 × 10⁶ cells/8 ml). The structural and functional integrity of these reaggregated cell cultures, including the absence of single or damaged cells, has been demonstrated [27]. Conditioned medium, collected on day 2 and day 5 or 6, was stored at –25 °C until used, after the aggregated cells had been removed by centrifugation at 100 g.

Preparation of a monoclonal anti-(rat PRL)–Sephacrose 4B column

A BALB/c mouse was immunized by repeated intraperitoneal injections with 10 µg of rat PRL (rPRL) (PRL-I-5, obtained from Dr. A. F. Parlow through the National Hormone and Pituitary Program, NIDDK, Bethesda, MD, U.S.A.) in either complete Freund's adjuvant (first injection) or incomplete Freund's adjuvant (booster injections 2 and 4 weeks after the first injection). At 3 days before cell fusion, a booster injection of 50 µg of rPRL (own preparation; extracted from rat pituitaries) was given in 0.9 % NaCl. Fusion of the mouse spleen cells with an SP2/0 myeloma cell line was performed according to the protocol of de St. Groth & Scheidegger [28]. The medium of growing hybridoma cells was tested for the presence of mouse monoclonal antibodies against rPRL by a solid-phase r.i.a. In short, hybridoma culture medium was transferred into star tubes (Nunc) coated with rabbit anti-(mouse IgG) (Dakopatts, Glostrup, Denmark). Then [¹²⁵I]PRL was added, and after washing away the unbound [¹²⁵I]PRL the tubes were counted for radioactivity in a γ-radiation counter. Hybridoma cells producing antibody were subcloned twice by limiting dilution. Ascites fluid was produced by peritoneal injection of 10⁶–10⁷ hybridoma cells into a BALB/c mouse according to standard methods. A partially

purified IgG fraction, prepared by ammonium sulphate (50 % saturation) precipitation followed by gel filtration chromatography on an AcA44 column (Pharmacia, Uppsala, Sweden), was coupled to CNBr-activated Sepharose 4B (Pharmacia).

Purification of PRL and PRL variants

About 1 litre of anterior pituitary cell conditioned medium, pooled according to animal model (immature female rats, adult male or female rats) and containing 5–15 mg of PRL immunoreactivity (PRL-ir), was loaded on to an anti-rPRL–Sephacrose 4B column (80 ml gel volume; IgG load of 300 mg). After extensively washing the column with phosphate-buffered saline (PBS, containing 0.5 M-NaCl, pH 7.4), the bound PRL was eluted with PBS (pH 3.5) and immediately neutralized with 2 M-Tris. PRL-ir in flow-through and eluate was monitored by r.i.a. (kit obtained from Dr. A. F. Parlow through the National Hormone and Pituitary Program: rPRL-I-5 for iodination, standard rPRL-RP-3 and rPRL-S-9 antiserum diluted 1:7500). Loss of PRL in the flow-through and during the wash step corresponded respectively to 0.2–0.3 % and 0.04 % of total PRL-ir loaded. Peak fractions were collected and concentrated with a Centriprep-10 concentrator (Amicon, Danvers, MA, U.S.A.). Final recovery of PRL-ir was 80–85 %. PRL variants were separated from 24 kDa PRL by anion-exchange chromatography on a Mono Q column (5 mm × 50 mm, Pharmacia). Loading buffer was 0.1 M-NH₄Cl, pH 9, and elution was with a slow linear gradient from 0.14 to 0.18 M-NH₄Cl, pH 9, in 40 min with a flow rate of 1 ml/min. After concentration of peak fractions with a Centricon-10 concentrator, further purification was by reversed-phase h.p.l.c. using a C₁₈ 30 nm microbore column (Delta Pak C₁₈, 2 mm × 150 mm; Millipore–Waters). A sample of 100–200 µg was loaded on to the column and eluted with water/acetonitrile, containing 0.1 % trifluoroacetic acid (TFA) (linear gradient 45–55 % acetonitrile in 50 min), at a flow rate of 0.25 ml/min. An inert h.p.l.c. system (600 E, Millipore–Waters) was used. Peaks were monitored by u.v. absorbance (Waters 490 E detector, Millipore) and PRL r.i.a.

Polyacrylamide-gel electrophoresis

Determination of the purity of the PRL variants and further characterization was carried out by SDS/PAGE (Phastsystem, Pharmacia) under reducing (2.5 % SDS/40 mM-dithiothreitol, 5 min at 100 °C) and non-reducing (2.5 % SDS, 5 min at 100 °C) conditions on 8–25 % polyacrylamide gradient gels (Phastsystem). Staining was according to an improved silver staining procedure (Phastsystem). Relative molecular masses were estimated by comparison with molecular mass protein standards (Pharmacia) using log molecular mass versus migration distance (*R_f*) plots.

For immunostaining, gels were blotted using a semi-dry electroblotting method [29] on a nitrocellulose membrane (0.2 µm; Schleicher and Schuell, Dassel, Germany). After saturation with 3 % normal swine serum (1 h, 45 °C) and overnight incubation with anti-rPRL antiserum (1:500, anti-rPRL-IC-4 from NIDDK), staining was obtained using an avidin–biotin–peroxidase detection system (see staining of tissue sections).

Determination of cleavage sites

The cleavage site of clPRL was determined by *N*- and *C*-terminal amino acid analysis and comparison with the known rPRL amino acid sequence [30]. The *N*-terminal sequence was determined by Dr. B. De Cock (Rega Institute, University of Leuven, Belgium) by loading 5 µg of purified clPRL on to an automated gas-phase sequencer (Applied Biosystems, Foster City, CA, U.S.A.). The *C*-terminal amino acids were determined

in our laboratory by enzymic degradation with carboxypeptidase Y (Sigma). In short, 5 μ g of purified clPRL-1 or 24 kDa PRL was incubated with 0.05 units of carboxypeptidase Y in 50 mM-sodium acetate buffer, pH 5.5. L-Norleucine was added as an internal standard. After 20 and 60 min, aliquots were taken and polypeptides were precipitated with a 4-fold excess of ice-cold acetone. Free amino acids were lyophilized, derivatized with phenylisothiocyanate (Pierce, Rockford, IL, U.S.A.) and separated by h.p.l.c. (Waters Pico-Tag system, Millipore).

Nb2 lymphoma cell PRL bioassay

Nb2 node lymphoma cells [31] were maintained as suspension cultures in Fisher's medium supplemented with 10% fetal calf serum (Gibco), 0.1 mM-2-mercaptoethanol, penicillin (35 mg/l) and streptomycin (50 mg/l) in an atmosphere of CO₂/air (1.5:98.5) at 37 °C. Then, 24 h before performing the bioassay, Nb2 cells were transferred to serum-free medium (SFM) consisting of Fisher's medium supplemented with 0.1 mM-2-mercaptoethanol, 1.5% BSA, 4 mg of linoleic acid/l, 1 mM-sodium pyruvate, 1.2 mg of human transferrin/l (Serva) and 25 nM-sodium selenite. In this medium the cells do not grow in the absence of lactogenic hormones. On the day of the bioassay, Nb2 cells were transferred to the wells of a microtitre plate at a density of 10⁴ cells/50 μ l of SFM. Standard PRL (NIDDK rPRL-B-6) and samples were diluted in SFM (100–1 pg of PRL/50 μ l), sterilized by filtration and added to triplicate wells containing Nb2 cells. After 72 h the Nb2 cell response was measured as described by Ealey *et al.* [32]. Briefly, intracellular NADH formation was assayed by adding 62.5 μ g of 3-(4,5-dimethylthiazol-2-yl)-2,5-di-phenyltetrazolium bromide (Janssen Chimica, Beerse, Belgium) in 25 μ l of PBS. After 1–2 h incubation at 37 °C, 60 μ l of acidified Triton X-100 was added and absorbances were read automatically at a test wavelength of 540 nm and a reference wavelength of 690 nm in a Titertek Multiskan MCC system (Eflab, Helsinki, Finland). The alternative of this test is the measurement of cell proliferation [31]. Using rPRL-B-6 (NIDDK) as a standard, both methods gave the same results, and the measurement of dehydrogenase activity was more sensitive (results not shown).

Treatment of anterior pituitary reaggregate cell cultures with PRL and clPRL variants, and immunostaining and autoradiography of [³H]thymidine-labelled cells

On day 4 of culture, bromocriptine (Sandoz, Basel, Switzerland), a potent dopamine agonist, was added to anterior pituitary cell aggregates from 14-day-old female rats at a final concentration of 10 nM in order to decrease basal PRL release, and 24 h later clPRL variants or 24 kDa PRL was added. After another 24 h [³H]thymidine (10 μ Ci/2 ml of culture medium; 100 Ci/mmol; Amersham) was added for 16 h in the presence of clPRL variants or 24 kDa PRL. Bromocriptine (10 nM) remained present during the entire test period.

After a 16 h incubation with [³H]thymidine, aggregates were fixed in Zamboni fixative (4% paraformaldehyde/15% saturated picric acid in phosphate buffer, pH 7.6) [33] and embedded in paraplast (Sherwood Medical Instruments, St. Louis, MO, U.S.A.) as previously described [34]. Sections of 4 μ m were cut on a Reichert–Jung microtome (Cambridge Instruments, Nussloch, Germany). After removal of the paraffin, sections were treated with trypsin (type III, Sigma) (0.1%, 5 min), preabsorbed with 20% normal swine serum (Dakopatts) and incubated overnight with the following antisera: anti-hACTH-IC (1:1600), anti-rPRL-IC-4 (1:3000), anti-rTSH-S-5 (1:1500), anti-rLH-S-10 (1:12500), anti-rFSH-S-11 (1:6250) (all from NIDDK), anti-(cow S-100 protein) (1:1000, Dakopatts) and anti-GH-IC (1:1000; Union Chimique Belge Bioproducts, Braine l'Alleud,

Belgium). Staining was obtained using biotinylated swine anti-rabbit IgG treated with avidin biotin/horseradish peroxidase (Dakopatts) and 5-amino-3-ethyl-carbazole. Immunostained sections were dipped in photographic emulsion (NTB2; Kodak) and developed 5 days later. Nuclei were counterstained with haematoxylin and slides were mounted in chrome/glycerine gel for light-microscopic examination. [³H]Thymidine-labelled nuclei present in the stained cells of each cell type were counted and expressed as percentages of the total number of labelled nuclei. In control cultures (without bromocriptine), [³H]thymidine labelled 12–16% of the cell nuclei. When after a 16 h labelling period [³H]thymidine was removed by repeated washing and the cultures were incubated for another 2 days in the absence of [³H]thymidine, the number of [³H]thymidine-positive cell nuclei increased to 30–35%, with weaker labelling of the nuclei, clearly indicating that in the present conditions [³H]thymidine incorporation is an indirect measure of cell mitosis.

Statistics

Statistical analysis was performed by Dr. E. Lesaffre (Biostatistical Centre, Department of Epidemiology, University of Leuven) using SAS software (SAS Institute, Cary, NC, U.S.A.). Dunnett's test was used to compare the ratio of biopotency to immunopotency of the PRL variants. As [³H]thymidine incorporation in specific cell types varied from culture to culture and a proportional change in [³H]thymidine incorporation among cultures for all treatment groups could be expected, logarithmic values of the scored numbers of [³H]thymidine-positive cells were used. Interaction between cell type and treatment was tested using analysis of variance with repeated measurements. Different treatments within one cell type were compared using Dunnett's tests.

RESULTS

Purification of PRL variants

PRL-like material from conditioned culture medium was first immunoabsorbed on an anti-rPRL–Sephacrose 4B column. A representative affinity purification experiment is shown in Fig. 1. PRL-ir was eluted in PBS, pH 3.5, as a sharp peak. After concentration, the mean recovery was 80% of loaded PRL-ir. This material was separated into three PRL-ir peaks by anion-exchange chromatography (peaks I, II and III; Fig. 2). Each of these components was then processed by reversed-phase h.p.l.c. While peak I and peak II were each eluted as one peak (Fig. 3), peak III was separated into three components (III₁, III₂ and III₃) (Fig. 3). Peaks I, II and III₃ had the same retention times. On comparing the amounts obtained by u.v. absorbance and by r.i.a., no marked differences in immunoreactivity between the different peaks was observed (Figs. 2 and 3). Rechromatography of peak III₃ by reversed-phase h.p.l.c. resulted in one peak with no change in retention time (Fig. 3).

The purity and the nature of the variants were determined by SDS/PAGE under reducing and non-reducing conditions. As shown in Fig. 4, there was no difference in apparent molecular mass between peaks I, II and III₃; they migrated as a single band with an estimated molecular mass of 23 kDa under non-reducing conditions and of 24 kDa under reducing conditions. Peak I represented 85–90% of total PRL as estimated by r.i.a., and is the major form of PRL. Peaks II and III₃ were considered as being charge variants of the major form of PRL. Peaks III₁ and III₂ each migrated as one band under non-reducing conditions (~24 kDa) (Fig. 4a), but after reduction they yielded two fragments. The first fragment from peak III₁ had an estimated molecular mass of 16 kDa whereas that from peak III₂ was 17 kDa (Fig. 4b). Unlike peak III₁, peak III₂ was only partially

Fig. 1. Affinity purification of PRL on an anti-rPRL-Sepharose 4B column

Conditioned culture medium (900 ml; reaggregate cell cultures of pituitaries from 14-day-old female rats; 8.1 mg of PRL-ir) was loaded on to the column. The flow-through contained 16.2 µg of PRL-ir (0.2% of loaded PRL-ir). Washing with 160 ml of PBS, pH 7.4 (arrow 1), resulted in loss of 3.5 µg of PRL-ir (0.04% of that loaded). PRL-ir was eluted by PBS, pH 3.5 (arrow 2), in a volume of 50 ml. After concentration on a Centriprep-10, 6.5 mg of PRL-ir was recovered (80%).

Fig. 2. Chromatography of affinity-purified PRL on a Mono Q anion-exchange column

Affinity-purified PRL (1.5 mg) was loaded on to a Mono Q column and eluted with 0.1–0.3 M-NH₄Cl, pH 9, at a flow rate of 1 ml/min. Peak I is 24 kDa PRL, peak II is a charge variant and peak III is a mixture of two cleaved and one charge variant (see Figs. 3 and 4).

purified. The second fragments of peaks III₁ and III₂ were not or barely visualized by silver staining of the gels (Fig. 4b). However, these fragments could be visualized by blotting the gel on nitrocellulose and immunostaining with anti-rPRL antiserum (Fig. 4c). Their molecular masses were around 10 kDa, but these estimations are probably less accurate as they moved near to the limit of the linear relationship between the log of the molecular mass and migration distance (Pharmacia Phastsystem Separation Technique File No. 110). Based on their behaviour on SDS/PAGE under reducing conditions, peak III₁ and the major component of peak III₂ were identified as cIPRL variants, with

Fig. 3. C₁₈ reversed-phase h.p.l.c. of the different peaks from the Mono Q anion-exchange column

The different peaks were loaded on a Delta Pak C₁₈ 30 nm reversed-phase column. A small fraction was loaded of peak I (45 µg). Peaks II and III were concentrated with a Centricon-10 microconcentrator; 25 µg of peak II and 72 µg of peak III was loaded. Elution was with water/acetonitrile, containing 0.1% TFA, at a flow rate of 0.25 ml/min. Peaks I and II gave one peak by reversed-phase h.p.l.c. Peak III was separated into three peaks: III₁ and III₂ are cIPRL variants; III₃ is a charge variant (see Fig. 4). Rechromatography of peak III₃ (37 µg) gave one peak.

peak III₁ hereafter named cIPRL-1 and peak III₂ named cIPRL-2. Fig. 4(b), lane 3 shows that these cIPRL variants were already present in peak III of the anion-exchange column, since SDS/PAGE of peak III under reducing conditions showed a double band of 16/17 kDa. The large fragment of cIPRL-2 had always a slightly larger molecular mass than the large fragment of cIPRL-1. Values estimated in three independent experiments were 16.8 and 15.8 kDa, 18 and 17 kDa and 17 and 16 kDa respectively.

All of these variants were consistently found in different batches of conditioned culture medium of reaggregate cell cultures from immature female, adult female and adult male rat anterior pituitary. The approximate amounts, estimated by integration of the peaks and expressed as a percentage of total PRL originally present, were as follows: peak II, 3–7%; peak III₁ (cIPRL-1), 0.6–1.1%; peak III₂ (cIPRL-2), 0.6–0.7%; peak III₃, 4–11%.

Cleavage site of cIPRL-1

When cIPRL-1 was analysed in a gas phase N-terminal sequenator, three fragments were found (Table 1 and Fig. 5). Fragment A represented about 100 pmol and had an amino acid sequence corresponding to 24 kDa rPRL-(1–20) [30] (only 20 N-terminal amino acids were determined). Fragment B represented 80–85 pmol and the sequence corresponded to rPRL-(146–165) [30]. Fragment C represented 15–20 pmol; it was identical to fragment B except that amino acid 146 was missing.

C-Terminal amino acids were determined by partial enzymic degradation with carboxypeptidase Y. When 5 µg of 24 kDa PRL was incubated with 0.05 units of carboxypeptidase Y no amino acids were released. This was expected, since the C-

Fig. 4. SDS/PAGE under non-reducing (a) and reducing (b, c) conditions of PRL variants separated on a Mono Q column and by C_{18} reversed-phase h.p.l.c.

Protein bands were visualized by silver staining (a, b); the small fragments expected after reduction of cPRL-1 and cPRL-2 were not seen with this staining procedure. They could be visualized by immunostaining of a Western blot with anti-rPRL-IC-4 (1:500, from NIDDK) (c). Lane 1, peak I of Fig. 2 (24 kDa PRL); lane 2, peak II of Fig. 2 (a charge variant of 24 kDa PRL); lane 3, peak III of Fig. 2 (a charge variant of 24 kDa PRL and cPRLs); lane 4, peak III₁ of Fig. 3 (cPRL-1); lane 5, first half of peak III₂ of Fig. 3 (cPRL-2 contaminated with cPRL-1; peak III₁); lane 6, second half of peak III₂ of Fig. 3 (cPRL-2 contaminated with a charge variant of 24 kDa PRL; peak III₃); lanes 7 and 8, peak III₃ of Fig. 3 (a charge variant of 24 kDa PRL); lane 9, molecular mass standards; lane 10, cPRL-1 fragments; lane 11, cPRL-2 fragments.

Table 1. Sequence and molar amounts of amino acids in cPRL-1 determined by automatic Edman degradation

The amounts and identities of phenylhydantoin (PTH)-amino acids were determined by comparing their elution peaks on reversed-phase h.p.l.c. with those of known amounts of standard PTH-amino acids (75 pmol). Contaminating amino acids were present in negligible amounts. The general decrease in amount from the first to the last cycle results from a loss of polypeptide. Molar amounts of amino acids were corrected for residual values of previous runs.

Cycle	Amino acids (pmol)	Position*		
		Fragment A	Fragment B	Fragment C
1	Leu (308), Val (13)	1 Leu	146 Leu	147 Val
2	Pro (76), Trp (14), Val (70)	2 Pro	147 Val	148 Trp
3	Ser (15), Trp (41), Val (49)	3 Val	148 Trp	149 Ser
4	Gln (12), Ser (64)	4 Cys†	149 Ser	150 Gln
5	Gln (51), Leu (16), Ser (50)	5 Ser	150 Gln	151 Leu
6	Gly (38), Leu (75), Pro (11)	6 Gly	151 Leu	152 Pro
7	Gly (40), Pro (44), Ser (10)	7 Gly	152 Pro	153 Ser
8	Asp (32), Leu (10), Ser (40)	8 Asp	153 Ser	154 Leu
9	Gln (7), Leu (60)	9 Cys†	154 Leu	155 Gln
10	Gln (67), Gly (10)	10 Gln	155 Gln	156 Gly
11	Gly (36), Thr (32), Val (6)	11 Thr	156 Gly	157 Val
12	Asp (8), Pro (10), Val (20)	12 Pro	157 Val	158 Asp
13	Asp (20), Leu (18)	13 Leu	158 Asp	159 Glu‡
14	Glu (25), Pro (14)	14 Pro	159 Glu	160 Glu
15	Glu (32), Ser (8)	15 Glu	160 Glu	161 Ser
16	Leu (12), Ser (18)	16 Leu	161 Ser	
17	Lys (11), Phe (8)	17 Phe	162 Lys	
18	Asp (11)	18 Asp	163 Asp	
19	Arg (8), Leu (14)	19 Arg	164 Leu	
20	Ala (7), Val (2)	20 Val	165 Ala	

* Amino acid sequences were compared with the known PRL sequence [30].

† Cysteine is not identifiable in this system.

‡ Not detected.

terminal amino acid of 24 kDa PRL, Cys, is part of a disulphide bridge. Incubation of 5 µg of cPRL-1 with 0.05 units of carboxypeptidase Y for different times (20 and 60 min) first released Tyr followed by Ile.

PRL-like bioactivity of PRL variants

To assess the PRL-like bioactivity of the PRL variants, the *in vitro* Nb2 lymphoma bioassay was used. As shown in Table 2, the ratio of biopotency to immunopotency of the charge variants was significantly lower than that of 24 kDa PRL, whereas the cleaved variants had the same biopotency as 24 kDa PRL. As indicated by SDS/PAGE under reducing conditions cPRL-2

(peak III₂) was slightly (~10%) contaminated with the charge variant peak III₃. Because this charge variant had a decreased ratio of biopotency to immunopotency, the higher biopotency/immunopotency ratio of peak III₂ cannot be the result of this contamination. The biopotency of pure cPRL-2 is probably slightly greater than indicated in Table 2.

Effect of PRL variants on [³H]thymidine incorporation into DNA in reaggregate anterior pituitary cell cultures of 14-day-old female rats

To investigate the effect of PRL variants on [³H]thymidine labelling of DNA in aggregates of 14-day-old female rat anterior

Fig. 5. Schematic diagram of PRL

Shown are the three disulphide bridges, the amino acid sequences revealed by *N*- and *C*-terminal sequence analysis and the cleavage site of cIPRL-1.

Table 2. Biopotency/immunopotency ratio of PRL variants

Biopotency was measured using the Nb2 assay and immunopotency was measured by r.i.a. Results are means $\pm$ s.e.m. ($n = 3$). * $P < 0.05$ compared with 24 kDa PRL (Dunnett's tests). cIPRL-2 (peak III₂) was contaminated with 5–10% of peak III₃.

Peak	Biopotency/immunopotency
I (24 kDa PRL)	0.93 $\pm$ 0.09
II (charge variant)	0.35 $\pm$ 0.03*
III ₁ (cIPRL-1)	1.01 $\pm$ 0.18
III ₂ (cIPRL-2)	0.95 $\pm$ 0.08
III ₃ (charge variant)	0.36 $\pm$ 0.02*

pituitary cells, we suppressed the possible effects of PRL and variants released by the cultures before and during testing by treating the cultures 24 h in advance and throughout the test period with the dopamine agonist bromocriptine (10 nM). Incubating these cultures with cIPRL-1 had a significant effect compared with 24 kDa PRL on the different cell types (Anova with repeated measurements, $P < 0.0001$). As shown in Fig. 6, the percentage of [³H]thymidine-labelled cells identified as gonadotrophs and thyrotrophs was significantly (Dunnett's test, $P < 0.05$) increased when cultures were incubated with cIPRL-1 (5–10 μ g/ml). No such increase was seen after treatment with 24 kDa PRL used in the same amounts. The effect of cIPRL-1 persisted at a dose as low as 0.5 μ g (about 20 nM) (two experiments; results not shown). [³H]Thymidine labelling of corticotrophs was increased by cIPRL-1 but also by 24 kDa PRL (Dunnett's test, $P < 0.05$); moreover, this effect was small and no difference was found between treatments. In two of the three experiments, the effect of partially purified cIPRL-2 (~10% contaminated with the charge variant peak III₃) was tested; it failed to affect [³H]thymidine incorporation in gonadotrophs and thyrotrophs.

Bromocriptine by itself decreased the number of [³H]thymidine-positive cells identified as lactotrophs (–42%,

$P < 0.05$) and corticotrophs (–15%, $P < 0.05$), and increased [³H]thymidine labelling of somatotrophs (+55%, $P < 0.05$) and folliculo-stellate cells (+65%, $P < 0.05$).

DISCUSSION

The present investigation shows that two cleaved forms of PRL exist in the rat anterior pituitary under physiological culture conditions, one of which has a cleavage site between Tyr-145 and Leu-146. Until now only one form of anterior pituitary cIPRL has been reported, and the cleavage site was not determined [8,16,20]. In these reports [8,16,20] rat anterior pituitary cIPRL was characterized only by means of SDS/PAGE. As the large fragments of the cIPRLs found here differ only slightly in molecular mass, previous studies could have overlooked the fact that the band around 16 kDa is a double band. We first purified PRL-ir molecules from conditioned culture medium using an anti-rPRL-Sepharose 4B column. This PRL-ir material was further analysed by anion-exchange and reversed-phase h.p.l.c. and finally by SDS/PAGE. We consistently found two cIPRLs which after reduction showed the same distinct fragments, the larger of which had a molecular mass, estimated by SDS/PAGE, of approx. 16 kDa (cIPRL-1) and 17 kDa (cIPRL-2) respectively. It is possible that the formation of a second form of cIPRL is favoured under our culture conditions, i.e. reaggregated AP cells in a serum-free medium. Other studies [14,17–19] dealt with the 16 kDa fragment of cIPRL or with cIPRL generated by incubating intact 24 kDa PRL with subcellular fractions of target tissues. Possibly only one form of cIPRL is formed in the target tissues.

Using *N*-terminal amino acid sequence analysis of PRL cleaved on incubation with mammary gland tissue, Vick *et al.* [14] reported that the *N*-terminal amino acid of the small fragment corresponded to amino acid 149 of the 24 kDa PRL molecule. However, this study could not definitely identify the exact cleavage site, since the *C*-terminal sequence of the large fragment was not determined, leaving open the possibility that amino acids may have been removed prior to position 149 by non-specific proteolysis during extraction and isolation. In the present study, *N*-terminal sequence analysis of cIPRL-1 purified from the conditioned medium of reaggregated AP cell cultures revealed that the *N*-terminal amino acid of the small fragment corresponded in 80–85% of cases to amino acid 146 of the 24 kDa PRL molecule and in about 15–20% of cases to amino acid 147. The *C*-terminal sequence of the large fragment, as determined by treatment with carboxypeptidase Y, corresponded to Ile-144–Tyr-145 of the 24 kDa PRL molecule. Combining the data from *N*- and *C*-terminal sequence analyses, we conclude that the cleavage site of cIPRL-1 of anterior pituitary origin is between amino acids 145 and 146. Amino acid 146 may have been partially removed by subsequent *N*-terminal proteolysis. The cleavage site of cIPRL-2 could not be determined, mainly because cIPRL-2 could not be purified in sufficient quantity. Nevertheless, the large fragment of cIPRL-2 was consistently found to be slightly larger than the large fragment of cIPRL-1, which means that the cleavage site of cIPRL-2 must be downstream of the cleavage site of cIPRL-1.

Vick *et al.* [14] reported that after cleavage of 24 kDa PRL by mammary gland tissue, the immunoreactivity of the molecule was decreased when measured with the NIDDK antisera anti-rPRL-S-6 and anti-rPRL-S-8, but was not markedly changed when measured with anti-rPRL-S-7 and anti-rPRL-S-9. In agreement with these authors, we observed no marked difference in immunological activity between 24 kDa PRL, cIPRL-1 and cIPRL-2, as measured by r.i.a. using NIDDK rPRL-S-9. Fur-

Fig. 6. Influence of clPRL-1 and 24 kDa PRL on [^3H]thymidine labelling of various cell types in reaggregate anterior pituitary cell cultures from 14-day-old female rats

AP cell aggregates, preincubated for 24 h with 10 nM-bromocriptine, were treated for 40 h (in the presence of 10 nM-bromocriptine) with clPRL-1 or 24 kDa PRL; during the last 16 h [^3H]thymidine was present. After fixation and embedding in paraplast, 4 μm sections were cut, immunostained and dipped in photographic emulsion. Folliculo-stellate cells were stained with anti-(cow S-100) serum, lactotrophs with anti-rPRL serum, somatotrophs with anti-(rat growth hormone) serum, thyrotrophs with anti-(rat thyroid-stimulating hormone) serum, gonadotrophs with a mixture of anti-(rat luteinizing hormone) and anti-(rat follicle-stimulating hormone) serum and corticotrophs with anti-(human adrenocorticotrophic hormone) serum. Values are expressed as the number of [^3H]thymidine-labelled cells staining for a particular hormone as a percentage of the total number of [^3H]thymidine-labelled cells counted. Results are means $\pm$ S.E.M. of three independent experiments; * $P < 0.05$ compared with control (Dunnett's test).

thermore, we did not observe a difference in PRL-like bioactivity between these molecules when measured in the Nb2 bioassay.

Casabiell *et al.* [20] have shown that a clPRL is produced as an artefact during tissue extraction and dialysis by acid proteases at low pH, but not at physiological pH, and have suggested that in previous work the presence of clPRL may have been an artefact because acid extraction procedures were used. In the present work, unlike in previous work, clPRLs were isolated from conditioned culture medium, i.e. medium in which anterior pituitary cell reaggregates, which closely resemble intact tissue [34–38], had been maintained for 2 or 3 days at physiological pH. Cleavage of PRL by intracellular acid proteases present in this starting material is most unlikely, as this material was collected and stored at pH > 7, and the presence of leaking damaged cells is negligible in these cultures (see the Materials and methods section). Furthermore, we have clearly shown that short-time exposure to acid conditions (pH 2) during reversed-phase h.p.l.c. did not produce the clPRLs. Indeed, as shown by SDS/PAGE, both clPRLs were already present in peak III (Fig. 2) after ion-exchange chromatography (pH 9) before application of the h.p.l.c. procedure. Reversed-phase h.p.l.c. of the other PRL-ir peaks obtained after ion-exchange chromatography also did not produce the clPRL forms. Finally, rechromatography of h.p.l.c. peak III₃ (Fig. 3) gave a single peak with the same retention time. The only other occasion of exposure to acid conditions was during elution of the PRL-like molecules from the anti-rPRL monoclonal antibody affinity column (pH 3.5). However, this exposure time was very short, and the eluted material was immediately neutralized. Moreover, before elution the column was extensively washed with PBS at pH 7.4, which should have removed the small amount of acid protease present.

The search for a physiological role for clPRL has, so far, been focused on peripheral target tissues, especially the mammary gland [12,14–19]. We demonstrate here a biological activity of clPRL-1 at its site of production (the anterior pituitary) at nanomolar concentrations. clPRL-1 specifically enhanced DNA synthesis in gonadotrophs and thyrotrophs from 14-day-old rats, an effect not caused by 24 kDa or clPRL-2. As we found that clPRLs are formed in the anterior pituitary in amounts only 0.6–1 % of the main (24 kDa) form of PRL, an action in distant

target tissues seems unlikely. We therefore suggest that clPRL-1 of anterior pituitary origin may have a local paracrine function within the pituitary itself. This hypothesis is feasible, as we have presented a body of evidence for the existence of functional intercellular communication in the anterior pituitary [26,35–37]. Using three-dimensional cultures of reaggregate AP cells, we showed that the hormone secretion of one anterior pituitary cell type can be influenced by other cell types in the gland. For example, PRL release stimulated by luteinizing-hormone-releasing hormone is mediated by a paracrine factor secreted by gonadotrophs [26]. Acetylcholine, synthesized in a subpopulation of corticotrophs, tonically influences the release of PRL and growth hormone [35]. Folliculo-stellate cells inhibit the secretory responses of various hormone-secreting cell types [36,37]. Recently we also showed that mitotic activity in the anterior pituitary is influenced by paracrine factors [39].

That a local paracrine function of clPRL-1 is a mitogenic function would agree with the biological actions of PRL-like molecules in general on distant target cells [16,17,21–23]. A selective action of clPRL-1 on gonadotrophs and thyrotrophs in the anterior pituitary of 14-day-old rats fits well with the time course of postnatal development of these cell types in the rat. At 1–2 weeks of age there is a transient accelerated growth of these cell types, in both size and number [40,41]. It would be of great interest to find out whether or not these two cell types lose their mitogenic response to clPRL-1 in older rats, and whether the production rate of clPRL-1 is transiently increased during postnatal life. Another intriguing question addresses the site where the cleavage of PRL takes place. Is PRL cleaved in the cell where it is produced (the lactotroph) before being released, or is it cleaved by its target cells in the anterior pituitary? The fact that 24 kDa PRL does not have the same biological activity as clPRL-1 in the cell aggregates strongly suggests that cleavage of PRL occurs in the lactotroph before secretion or immediately after release before the molecule reaches the target cells or clPRL-1 receptors within the anterior pituitary. Recently we found that LHRH and neuropeptide Y exert mitogenic effects on anterior pituitary lactotrophs from 14-day-old rats, an effect which is mediated by a growth factor released from gonadotrophs ([39]; D. Tilemans, M. Andries & C. Denef, unpublished work).

Thus there seems to exist a bidirectional interaction between gonadotrophs (belonging to the basophilic cell lineage) and lactotrophs (belonging to the acidophilic cell lineage) in the anterior pituitary, a phenomenon with potential importance in the fine control of reproduction. Interestingly, there is a morphological correlation of this interaction, in that lactotrophs and gonadotrophs display a topographical affinity for each other in the intact anterior pituitary as well as in our aggregate cell cultures [34].

In conclusion, we have demonstrated the existence of two different forms of cPRL secreted by reaggregated rat anterior pituitary cells. We have also determined the cleavage site of cPRL-1 and provided evidence that cPRL-1 of anterior pituitary origin can have specific, possibly paracrine, mitogenic actions on selective pituitary cell types.

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