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The addition of nine residues at the C-terminus of human prolactin drastically alters its biological properties

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We have added nine extra residues to the C-terminal of human prolactin and analysed the effect of this mutation on the ability of the hormone to bind to its lactogenic receptor and to induce Nb2 cell division. Both properties are markedly affected when compared to the natural 23-kDa human prolactin. Since no alteration of the global protein folding was detected either by circular dichroism or by infrared spectroscopy, the decrease in biological potency can be exclusively attributed to an effect of the nine additional residues on their near environment. From infrared analysis and secondary structure prediction, the elongated tail is assumed to be involved in a β-sheet with a few residues initially belonging to the fourth helix. Moreover, from the X-ray structures of porcine and human growth hormones, two proteins homologous to prolactins, the nine extra residues are likely to fold within a concave pocket delimited by helices 1 and 4, and the second half of the loop connecting helices 1 and 2 (loop 1). Thereby, we suggest that the additional residues prevent some residues belonging to this pocket from interacting with the lactogenic receptor. This is in perfect agreement with our earlier proposal that the binding site of prolactin to the lactogenic receptor is homologous to that of growth hormone to the somatogenic receptor, i.e. essentially composed of residues belonging to this concave pocket.

Prolactin (PRL), a 23-kDa pituitary-secreted hormone, is involved in more than 85 biological functions in vertebrates. These are mainly related to lactation, reproduction, growth, osmoregulation and immunomodulation (Nicoll and Bern, 1972; Clarke and Bern, 1980). The hormonal signal is mediated by specific membrane receptors, or lactogenic receptors (Boutin et al., 1988; Edery et al., 1989), which are found in the numerous target tissues (Kelly et al., 1991). Several studies, aimed at determining which region of PRL interacts with the receptor, have been reported. Sequence comparisons (Nicoll et al., 1986; Luck et al., 1989), chemical modifications (Doonen and Bewley, 1979; Andersen and Ebner, 1979; Necessary et al., 1985; de la Llosa et al., 1985; for review, see Nicoll et al., 1986) or mutational studies (Luck et al., 1989, 1990, 1991; Goffin et al., 1992) characterized some structural features potentially involved in the biological properties of PRL but none of these data unequivocally localized the global PRL binding site.

PRL belongs to a protein family also including growth hormone (GH) and placental lactogen. These hormones are evolutionarily related (Miller and Eberhardt, 1983) and share several structural, immunological and biological properties. To date, only the three-dimensionnal (3D) structures of porcine GH (pGH) and human GH (hGH) have been solved by X-ray diffraction (Abdel-Meguid et al., 1987; de Vos et al., 1992). These proteins fold in an antiparallel four-helix bundle with a characteristic up-up-down-down connectivity (Abdel-Meguid et al., 1987). Given the sequence similarity within the PRL-GH family, this GH folding pattern is likely to be shared by all the members of the protein family. Through an extensive mutational study, Cunningham and his coworkers (Cunningham et al., 1989; Cunningham and Wells, 1989) have recently identified the binding site I of hGH to the somatogenic receptor (Leung et al., 1987) as formed of three segments, namely parts of helices 1 and 4 and the second half of the long loop connecting helices 1 and 2 (loop 1). Although discontinuous in the sequence, these three regions are contiguous on the folded protein and form a compact patch interacting with the receptor. The further X-ray analysis of the hGH–hGH-binding-protein complex (de Vos et al., 1992) allowed a better understanding of the hormone–receptor interaction and confirmed the existence of a second binding site as previously suggested by the mutational approach (Cunningham et al., 1991).

Since, on one hand, the three segments constituting the binding site I of hGH are highly conserved within the PRL-GH family (Nicoll et al., 1986), and, on the other hand, the extracellular domains of the lactogenic and somatogenic receptors share several segments of more than 70% identity (Kelly et al., 1991), one can assume the binding site of the PRL to be very similar, if not identical, to that described for hGH. This is in total agreement with our recent findings that some residues belonging to the loop 1 of hPRL are also essential for maintaining the biological properties of the hormone (Goffin et al., 1992). Nevertheless, involvement of

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Abbreviations. PRL, prolactin; GH, growth hormone; h, human; p, porcine; FTIR, Fourier-transform infrared spectroscopy; 3D, three dimensional.

other regions, such as helices 1 and/or 4, has never been clearly demonstrated and the global shape of the PRL binding site remains unknown.

In this paper, we report structural and biological analyses of a hPRL mutant carrying nine extra residues at the C-terminus. From the structure/function studies of hGH, the C-terminal loop is at the edge of the hGH binding site I (Cunningham et al., 1989; Cunningham and Wells, 1989; de Vos et al., 1992). Otherwise, it is maintained close to helix 4 through a disulfide bridge between Cys189 (C-terminal loop) and Cys182 (helix 4)(Abdel-Meguid et al., 1987; de Vos et al., 1992). Hence, if the global shape of the PRL binding site is similar to that of hGH, one could expect the nine additional residues of this mutated hPRL to interfere with the hormone–receptor interaction and to alter the biological properties of the hormone.

MATERIALS AND METHODS

Materials

Restriction enzymes and ligase were purchased from Boehringer Mannheim (Germany), Amersham International (UK) and BRL (USA). Iodogen and bovine γ-globulin were purchased from Sigma (USA) and carrier-free $Na^{125}I$ was obtained from Amersham International (UK). *Taq* polymerase was provided by Cetus (USA). Rabbit antiserum to hPRL was from UCB (Belgium) and goat anti-rabbit from Gamma SA (Belgium). Purification of hPRL was performed using a column (100×2.6 cm) of Sephadex G-100 (Pharmacia). Culture medium and sera were purchased from Gibco (USA).

Mutagenesis

The mutation of hPRL stop codon (TAA) to Gln (CAA) occurred unexpectedly during a routine polymerase chain reaction experiment performed to introduce a single mutation (Goffin et al., 1992) in the hPRL cDNA (Cooke et al., 1981). It was detected by DNA sequencing. The next stop codon on the same reading frame (TGA) is nine triplets further; this leads to the extension of the protein C-terminus by the nine following amino acids: Gln200, Ala201, His202, Ile203, His204, Phe205, Ile206, Tyr207, Phe208. On account on its higher molecular mass (24 kDa), this hPRL mutant is thus called 24-kDa hPRL.

The *Nde*I–*Hin*dIII mutated fragment of hPRL cDNA, containing the entirety of the 24-kDa hPRL coding sequence, was restricted at *Nde*I and *Hin*dIII sites and reinserted in the pT7L expression vector (Paris et al., 1990; Goffin et al., 1992).

Expression and purification of hPRL

Expression and purification stages have been extensively described previously (Paris et al., 1990). Briefly, recombinant 23-kDa and 24-kDa hPRL were overexpressed in *Escherichia coli* as insoluble aggregates, at a yield around 150 mg hPRL/l culture. These inclusion bodies were denaturated in 8 M urea, 1% 2-mercaptoethanol, 0.2 M sodium phosphate pH 7, and solubilized proteins were allowed to refold through a 72-h dialysis against 50 mM NH_4HCO_3 pH 8. Finally, renaturated hPRL was loaded on a Sephadex G-100 column in order to separate monomers and multimers formed upon the renaturation step. Purified proteins were lyophilized for at least 24 h and conserved at 4°C.

Quantification of hPRL

Proteins were quantified by weighing the lyophilized powder on a precision balance (Electrobalance, Cahn 26) and by protein measurements following the Bradford method (1976). Disparity between weight and chemical measurements never exceeded 10%.

Characterization of hPRL

SDS/PAGE

Protein size and purity were assessed by SDS/PAGE in reducing conditions (2-mercaptoethanol) according to Laemmli (1970). Electrophoresis was performed for 1 h at 150 V and gels (15%) were stained with Coomassie blue.

Western blotting

After migration on SDS/PAGE, proteins were transferred (90 min, 250 mA) to a nitrocellulose filter using a Transblot Cell apparatus (Bio-Rad). The filters were then treated with polyclonal rabbit anti-hPRL serum (1 h, 37°C) followed by a goat anti-rabbit preparation coupled with peroxidase (1 h, 37°C). Final colour development occurred on addition of H_2O_2 and horseradish peroxidase color development reagent (Bio-Rad). The reaction was stopped with 5% SDS.

Circular dichroism

Lyophilized proteins were resuspended in 50 mM NH_4HCO_3 at a concentration of 200–500 µg/ml. Spectra were measured using a Jobin-Yvon dichrograph V linked to an Apple microcomputer for data recording and analysis. Ten measurements within the ranges 195–260 nm and 240–330 nm were made for each protein using a 0.1-cm pathlength quartz cell. Helicity was calculated at 222 nm (Chen et al., 1972).

Fourier-transform infrared spectroscopy

Attenuated total reflectance spectra were obtained on a Perkin Elmer 1720X FTIR spectrophotometer equipped with a liquid nitrogen-cooled MCT detector, at a resolution of 4 cm^{-1}, by averaging 128 scans. Protein samples (500 µg/ml) were dialysed for 24 h against 1 mM Tris/HCl pH 8; 50 µl of each hPRL sample were layered on a germanium crystal and dried under nitrogen to form a hydrated film. The internal reflection germanium crystal (50×20×2 mm, Harrick) with a aperture angle of 45°C yields 25 internal reflections. Every four scans, reference spectra of a clean germanium plate were automatically recorded and ratioed against the recently run sample spectra by an automatic sample shuttle accessory. The spectrophotometer was continuously purged with dry air. Helix and β-sheet content were estimated as described (Goormaghtigh et al., 1990).

Nb2 cell culture and *in vitro* bioassay

Human 23-kDa and 24-kDa PRL were assayed for lactogen activity by measuring their ability to stimulate the growth of lactogen-dependent Nb2 lymphoma cells (Gout et al., 1980) following the procedure of Tanaka et al. (1980). Cells were cultured in Fischer's medium containing 10% horse serum and 10% fetal calf serum; 24 h before the bioassays, cells were carefully centrifuged and resuspended in pre-

incubation medium containing only 1% fetal calf serum to reduce cell growth. Bioassays were performed in medium containing no fetal calf serum.

Different amounts of hPRL samples, diluted in phosphate-buffered saline, 0.1% bovine serum albumin, were added to 2.5 ml cells (approximately 10^5 cells/ml) plated in six-well Falcon plates. Each protein was assayed in duplicate at eight concentrations selected to induce detectable cell growth. Nb2 cells were counted after 3 days using a Coulter counter (Coulter Electronics Ltd, England).

Iodination of hPRL

Recombinant 23-kDa hPRL was iodinated by the iodogen method (Salacinski et al., 1981). 20 µg hPRL in 0.5 M sodium phosphate pH 7.4 was transferred to a borosilicate glass coated with 10 µg iodogen. The reaction was initiated by addition of 1 mCi carrier-free $Na^{125}I$. After 6 min at room temperature, the reaction was stopped by transferring the entire reaction mixture to a column (1 × 30 cm) of Sephadex G-100 equilibrated in 0.05 M sodium phosphate pH 7.4 containing 2% bovine serum albumin. Purified monomeric hPRL recovered after purification had a specific activity of 40–50 µCi/µg.

Binding experiments

Studies on the binding of the 23-kDa and 24-kDa hPRL to the lactogenic receptor were performed on Nb2 cell homogenates in order to avoid any internalization or degradation of iodinated hPRL by intact cells. Nb2 cells were synchronized for 24 h in Fischer's medium in the absence of fetal calf serum to reduce occupancy of PRL receptors. Cells were pelleted, resuspended in the same medium at a concentration of 10^8 cells/ml and homogenized by freezing and thawing followed by a short sonication. Aliquots of homogenates were frozen at −70°C for subsequent use in binding assays.

The assay conditions were described previously (Goffin et al., 1992). Homogenates, equivalent to 3×10^6 cells, were transferred to Eppendorf tubes and incubated for 16 h at 25°C with 40000–50000 cpm ^{125}I-hPRL in the presence of increasing amounts of unlabelled 23-kDa or 24-kDa hPRL (the final reaction volume was 0.5 ml). The assay was terminated by addition of 0.5 ml ice-cold buffer (0.025 M Tris/HCl, 0.01 M $MgCl_2$, 0.2% bovine γ-globulin, pH 7.5) followed by centrifugation (5 min, 11000 × *g*). The supernatant fraction was removed carefully and the pellets were counted in a gamma counter (Hybritech 002011B, Belgium).

Binding measurements were performed in duplicate in three separate experiments. Specific binding was calculated as the difference between radioactivity bound in the absence and in the presence of 2 µg unlabelled 23-kDa hPRL. Data are presented as the percentage of this specific binding. Competition curves were analyzed using the LIGAND PC program (Munson and Rodbard, 1980).

Secondary structure prediction

The conformation of the elongated C-terminus tail of 24-kDa hPRL has been predicted using three secondary structure prediction algorithms (Chou and Fasman, 1978; GOR method of Garnier et al., 1978; COMBINE method of Biou et al., 1988).

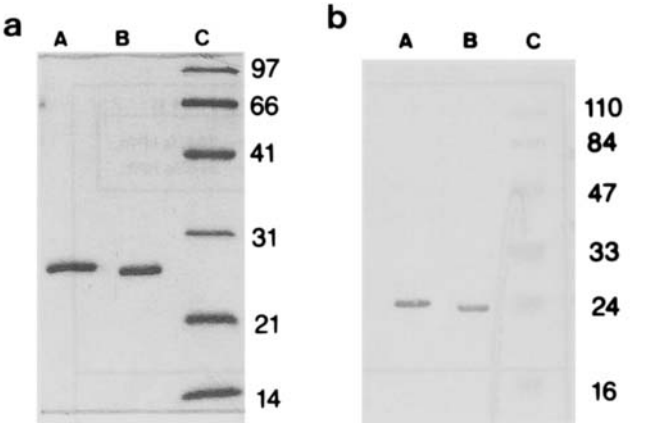

Fig. 1. Polyacrylamide gel (15%) electrophoresis (a) and Western blot analysis (b) of 23-kDa and 24-kDa hPRL. Before electrophoresis (1 h, 150 V), the proteins were boiled for 5 min in sample buffer containing 2-mercaptoethanol. Lanes A, B and C represent 24-kDa hPRL, 23-kDa hPRL and molecular mass markers (values in kDa), respectively.

RESULTS

Production and characterization of 23-kDa and 24-kDa hPRL

Protein productions and purifications were carried out as previously described for the recombinant 23-kDa hPRL (Paris et al., 1990; Goffin et al., 1992). The expression level of 24-kDa hPRL was similar to that of the unmodified hPRL (around 100 mg insoluble inclusion bodies/l culture). After one denaturation and renaturation cycle, a gel filtration (Sephadex G-100) purification step allowed the recovery of around 15 mg 23-kDa or 24-kDa hPRL/l culture. As shown in Fig. 1a, the purified fractions are highly enriched in hPRL. Both proteins were tested with a polyclonal anti-hPRL antibody preparation. Fig. 1b indicates that the 24-kDa hPRL reacts with the antibodies in a manner similar to the 23-kDa hPRL.

Conformation of the proteins was assessed by circular dichroism (CD; Fig. 2) and Fourier-transform infrared spectroscopy (FTIR).

In the far-ultraviolet range (195–260 nm), both proteins exhibit CD spectra characteristic of polypeptides containing residues primarily in α-helical conformation, with two minima at 208 nm and 222 nm and a maximum around 195 nm (Fig. 2a). The helicity calculated from the ellipticity at 222 nm according to Chen et al. (1972) is around 55% for 23-kDa hPRL and 50% for 24-kDa hPRL. A similar difference is obtained by FTIR, although the absolute percentage of helical structures is slightly under-estimated when compared to CD measurements (around 50% and 45% for 23-kDa and 24-kDa hPRL, respectively). FTIR also shows that the absorbance of the 24-kDa hPRL is significantly increased at 1631 cm^{-1}, indicating that 4% of its amino acids are in β conformation (data not shown). No β structures were detected for the 23-kDa hPRL. Finally, the CD spectrum of both hPRL are also markedly different in the near-ultraviolet range (240–330 nm; Fig. 2b). The positive CD bands in the 240–290-nm range are higher for 24-kDa hPRL than for the 23-kDa hPRL and curves cross the baseline towards negative bands at 248 nm and 241 nm, respectively.

Bioactivity of 24-kDa hPRL

Bioactivity of the hPRL was estimated by the ability to stimulate proliferation of rat lymphoma Nb2 cells whose

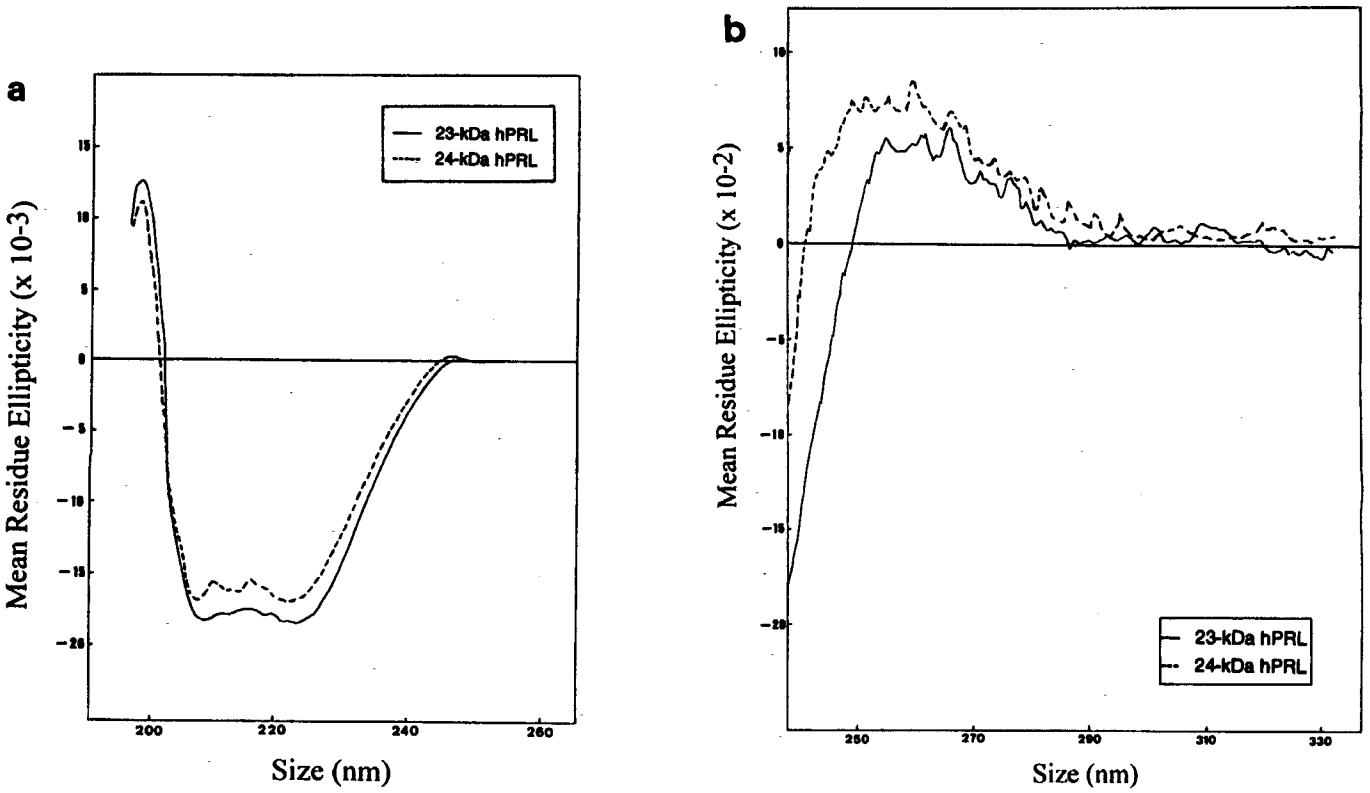

Fig. 2. Circular dichroic spectra in far ultraviolet (a) and near ultraviolet (b) of 23-kDa hPRL (——) and 24-kDa hPRL (---). Values are expressed as mean residue mass ellipticity (in deg. × $cm^2 \times dmol^{-1}$). Lyophilized proteins were resuspended in 50 mM ammonium bicarbonate, pH 8. Spectra were measured in a 0.1-cm path-length quartz cell. Helix content was calculated at the 222-nm minimum according to Chen et al. (1972).

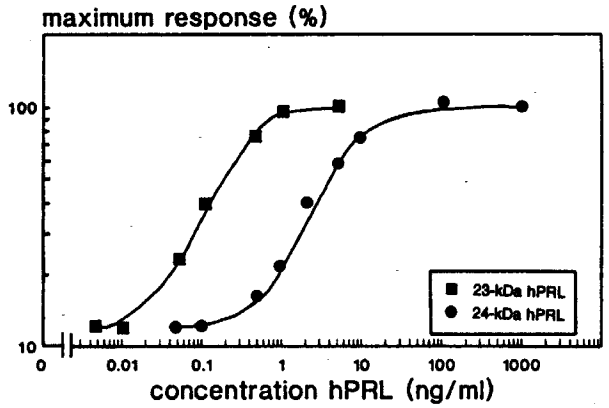

Fig. 3. Nb2 cell proliferation in the presence of increasing amounts of 23-kDa or 24-kDa hPRL. In this experiment, half-maximal growth was achieved by addition of 170 pg 23-kDa hPRL/ml and 5 ng 24-kDa hPRL/ml.

growth is lactogen-dependent (Tanaka et al., 1980). Since recombinant 23-kDa hPRL behaves similarly to pituitary-purified hPRL (Goffin et al., 1992), it was used as reference in both bioactivity and binding assays (see further). Fig. 3 represents Nb2 cell growth in the presence of increasing amounts of recombinant 23-kDa and 24-kDa hPRL. In all experiments, half-maximal cell growth occurred at 200 ±40 pg of 23-kDa hPRL/ml culture. Bioactivity of 24-kDa hPRL was estimated as the ratio between the amount of 23-kDa hPRL compared to 24-kDa hPRL necessary to produce half-maximal cell growth. In three separate experiments, bioactivity of 24-kDa hPRL was calculated as 3.3%, 1.6% and 1% that of 23-kDa hPRL.

Binding studies

Binding affinities of the hPRL to the lactogenic receptor were measured on Nb2 cell homogenates. The affinity of the 23-kDa and 24-kDa hPRL for the lactogenic receptor was estimated by their ability to compete for the binding of labelled 23-kDa hPRL to Nb2 cell homogenates (Fig. 4). The concentration achieving 50% displacement of 23-kDa ^{125}I-hPRL (IC_{50}) was between 2–4 ng/ml for 23-kDa hPRL, whereas it was 25–30-fold higher for 24-kDa hPRL.

Secondary structure prediction

As expected, structure prediction of 23-kDa and 24-kDa hPRL were indistinguishable in their common sequences (data not shown). The predicted fourth helix extremity was not shifted (Leu189). Whatever the algorithm used, the nine extra residues of 24-kDa hPRL (residues 200–208) were predicted to be essentially in β-strand or coil conformation (Fig. 5).

DISCUSSION

When the mutational approach is used to elucidate the role of some amino acids in the biological properties of a protein, it is of prime importance to verify that the residue substitution (or deletion) generates no modification of the

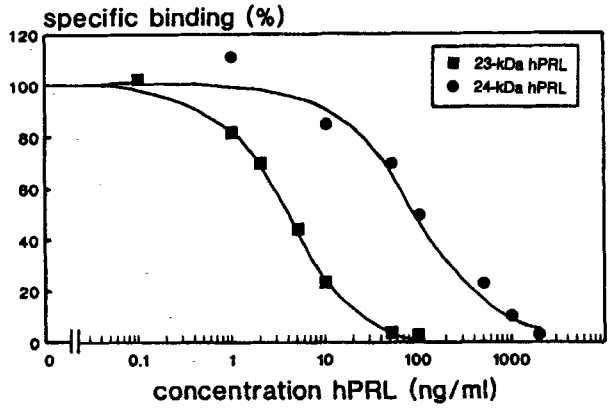

Fig. 4. Competitive binding curves of 23-kDa ^{125}I-hPRL (tracer) and unlabelled 23-kDa and 24-kDa hPRL (competitor). Binding of the tracer in the absence of competitor was taken as 100% binding. Non-specific binding was determined by addition of 2 μg unlabelled 23-kDa hPRL. Each point was performed in duplicate. In this experiment, concentration of competitor displacing 50% of the tracer (IC_{50}) was 4 ng/ml for 23-kDa hPRL and 100 ng/ml for 24-kDa hPRL.

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          helix 4                    elongation

<--------------------------->      <-------->

 170       180       190       200     208     NUMBERS

LSAYYNLLHCLRRDSHKIDNYLKLLKCRIIHNNNCQAHIHFIYF    24-kDa hPRL

HHHHHCBHBBCCCCCCCCCCHHHHHHCCCBCCCCCCCCHBBBBCC   PREDICTION
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Fig. 5. Secondary structure prediction of the C-terminal region of 24-kDa hPRL performed using the COMBINE method of Biou et al. (1988). The putative extremities of hPRL helix 4 are deduced from pGH and hGH X-ray structures. The nine extra residues of 24-kDa hPRL are underlined and labelled 'elongation'. First line represents residue numbers, second line the C-terminal amino acid sequence of 24-kDa hPRL, and third line the predicted conformation (H = α helix, B = β strand, C = coil).

global protein structure that could be more responsible for an alteration in the biological behavior than the mutation *per se*. As an example, Luck et al. (1990) observed a dramatic decrease of the Nb2 mitogenic effect of bovine PRL after the single deletion of Tyr28 whereas any residue substitution at this position was much less effective. Since Tyr28 belongs to an α-helix, its removal modifies the register of the whole helical segment, disturbs the global protein folding and, hence, is very likely to alter the functional properties of bPRL. Otherwise, the residue itself should not be a major binding determinant since it can be substituted by any other amino-acid without significant loss of bioactivity.

The global shape of 24-kDa hPRL is not altered since its elution volume on a molecular sieve during the purification procedure was indistinguishable from that of 23-kDa hPRL. Since the PRL/GH are all α proteins, far-ultraviolet circular dichroism (CD) is a good tool for estimating their content of secondary structures (Bewley and Li, 1972; Goffin et al., 1992). The far-ultraviolet CD spectrum of 24-kDa hPRL is similar to that of the native 23-kDa hPRL, with the two minima at 208 nm and 222 nm, characteristic of polypeptides with high helical content. The amount of α-helical structures was estimated to be 55% for 23-kDa hPRL and 50% for 24-kDa hPRL. A similar difference between both proteins (around 5%) was observed by FTIR. The slightly lower helical content calculated for the 24-kDa hPRL could be partially related to the addition of nine residues which decreases the overall average of helical content. Although a small loss of α-helical structures in the 24-kDa hPRL cannot be ruled out from these experiments, it appears nevertheless that the elongation of the C-terminal tail by addition of nine residues has no effect, or at least no detectable effect, on the global folding of the protein.

We observed more important differences between the CD spectra of 23-kDa and 24-kDa hPRL in the 240−330-nm range. These can be related to the presence, in the elongated C-terminus, of aromatic residues (Phe205, Tyr207 and Phe208), increasing the CD signal in this wavelength range. Similar changes of the near-ultraviolet CD spectrum have also been previously reported after deletion or mutation of Trp in hGH (Nishikawa et al., 1989).

Without X-ray or NMR data, it is obviously impossible to determine the actual 3D structure of the nine extra residues extending the C-terminus of 24-kDa hPRL. The three secondary prediction algorithms we used (Chou and Fasman, 1978; Garnier et al., 1978; Biou et al., 1988) all suggest that the elongated C-terminus of 24-kDa hPRL presents β-strand and coil conformations. This is relevant to the data obtained by FTIR from which nearly 4% of the amino acids of the 24-kDa hPRL are in β conformation. Although very weak, such an increase of the β-sheet content can be considered as significant since obtained from comparison of the original spectra, before Fourier transform and curve fitting that can introduce artefacts. The increase of β structures in the mutant is comcomitant to a similar decrease of the α-helical content (−5%, see above). One can thus assume that some residues initially involved in the fourth helix of 23-kDa hPRL adopt a β structure in the 24-kDa hPRL to form a small β-sheet with some residues of the elongated C-terminus (Ile203−Ile206 from Biou prediction). Two β-strands of four or five residues could thus account for the 4% of β structures calculated from the analysis of FTIR spectra. Such local modification of the structure could also be responsible for the small shift in wavelength detected in the 200−240-nm range of the CD spectrum.

A possible location for this elongated tail can be proposed from the analysis of the general folding of the PRL/GH proteins (Abdel-Meguid et al., 1987; de Vos et al., 1992), illustrated in Fig. 6. From this model, the C-terminus folds in a coil segment of six (PRL) to eight (GH) residues, maintained near helix 4 by a disulfide bridge (Cys191−Cys199 in hPRL, Cys182−Cys189 in hGH). Otherwise, the N-terminal part of helix 1, the C-terminal part of helix 4 and the second half of loop 1 delimit a pocket with a concave shape (de Vos et al., 1992). Hence, we propose that the nine extra residues of 24-kDa hPRL fold very near, or even within, this concave cavity. Such a hypothesis is in agreement with the overall length of nine residues as well as with their highly hydrophobic character (Ile203, Phe205, Ile206, Tyr207, Phe208), that makes them candidates for being buried rather than exposed at the surface of the protein.

Taken together, experimental and theoretical data suggest that the entire elongated tail of 24-kDa hPRL folds in the environment of the 'helix-1−helix-4−loop-1' concave cav-

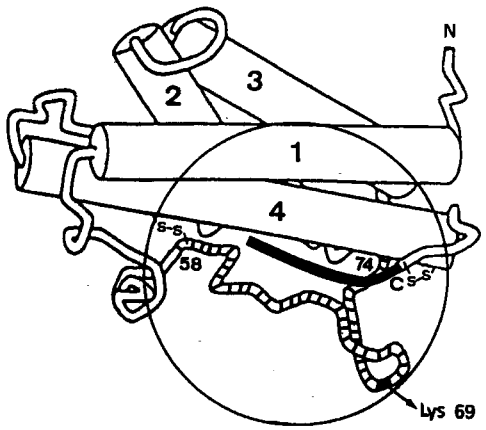

Fig. 6. Schematic view of the PRL/GH three-dimensional folding (Abdel-Meguid et al., 1987). Helices are represented by cylinders and labelled from 1 to 4. Disulfide bridges are represented by S-S. The circle localizes the binding site of hGH to its somatogenic receptor (Cunningham et al., 1989). The 58−74 loop region, involved in hPRL binding (Goffin et al., 1992), is striated. The position of Lys 69, a major binding determinant of hPRL, is roughly indicated. A possible location for the nine additional residues at the C-terminus of 24-kDa hPRL is shown in black.

ity, with a few of the nine extra residues being involved in a β-sheet with some residues initially belonging to the fourth helix of the wild-type 23-kDa hPRL.

Functional properties of the 24-kDa hPRL were estimated by measuring its ability, first, to bind to the lactogenic receptor of Nb2 cells and, second, to induce Nb2 cell division (Tanaka et al., 1980; Gout et al., 1980). Nb2 cells are a rat lymphoma cell line that is lactogen-dependent for growth; thus, it provides a reliable bioassay widely used for evaluating biological potency of lactogenic hormones (Luck et al., 1989, 1990, 1991; Davis and Linzer, 1989; Paris et al., 1990; Goffin et al., 1992). It has been previously reported that receptor binding of mutated lactogenic proteins is not necessarily a sufficient event for promoting Nb2 cell division (Davis and Linzer, 1989). In the case of the 24-kDa hPRL, both its binding affinity and mitogenicity are reduced by 50−100-fold compared to the 23-kDa hPRL. This leads to the conclusion that the lower mitogenic effect of the mutant results essentially from a lower binding affinity rather than from any alteration of the signal transduction process.

Since the global fold of the hormone is likely to remain virtually unchanged after the elongation of the C-terminus (see above), one can assume that these modifications of the biological behavior are directly correlated with the structural modifications occurring in the environment of the nine additional residues. In other words, the mutation is likely to affect mainly the binding site of hPRL to its receptor.

In hGH, most of the binding determinants involved in the binding site I belong to the concave pocket delimited by segments of helix 1, helix 4 and loop 1 (Cunningham and Wells, 1989; de Vos et al., 1992). The 3D structure of the hGH−hGH-binding-protein complex also shows that binding site I is mostly buried upon the hormone−receptor interaction (de Vos et al., 1992). We have recently proposed the binding site of PRL to be similar to that described for hGH, and we have partly confirmed this hypothesis by identifying some residues belonging to loop 1 of hPRL as binding determinants (Goffin et al., 1992). In the present work, we have hypothesized that the nine extra residues of 24-kDa hPRL fold within (or very near) the 'helix-1−helix-4−loop-1' cavity. This proposal, correlated with the lower biological potency of the mutant, suggests this concave pocket to be involved in the binding site of hPRL. This is therefore relevant to what has been demonstrated for hGH and strengthens our earlier hypothesis.

There may be two different ways by which the nine extra residues alter the biological properties of the 24-kDa hPRL. On one hand, by its steric hindrance, the elongated tail probably prevents some binding determinants belonging to the concave pocket from interacting more tightly with the receptor. At first sight, the residues whose binding is affected can be located in either of the three segments constituting this binding cavity (loop 1, helices 1 and 4). On the other hand, the probable conformational modifications occurring in the fourth helix (local replacement of α structures by β structures), as a direct consequence of the potein elongation, could prevent some residues specifically belonging to this helix from interacting with the receptor. This latter proposal does not question our hypothesis concerning the biological importance of the concave pocket since the fourth helix belongs to this binding cavity. It may in fact underline the functional involvement of that particular helical segment.

In the 58−74 region (loop 1), we have recently identified Cys58, Pro66 and Lys69 as three major binding determinants (Goffin et al., 1992). The role of Cys58 is essentially structural, in that the disulfide bridge it forms with Cys174 maintains the whole loop 1 in an appropriate conformation for a tight binding to the receptor. From the GH fold (Abdel-Meguid et al., 1987; de Vos et al., 1992), Pro66 is in the interior of the binding cavity; since the pyrollidine ring of Pro residues places strong conformational constraints on the backbone, the role of that residue could also be essentially structural. These observations suggest that Cys58 and Pro66 are not directly involved in a contact with the receptor through their side chains, but rather participate in maintaining the global shape of the binding site of hPRL. Hence, the steric hindrance of the elongated tail of 24-kDa hPRL has probably no, or only very low, effect on the functional role of these two residues. The third binding residue of this segment, Lys69, is located at the edge of the concave pocket (Fig. 6). It is fully exposed to the solvent and is in a conformation which should be favorable for direct interaction with a docking receptor molecule. From the location we proposed for the elongated tail of 24-kDa hPRL (Fig. 6), the nine additional residues should not interfere with the Lys69, even if some structural disturbance cannot be totally excluded. In conclusion, we think that the reduced bioactivity of 24-kDa hPRL cannot only result from a steric effect of the elongated tail on loop 1 residues, but is rather a consequence of a more global alteration of the whole hPRL binding site.

Thus, from these observations, we propose that some regions of helices 1 and/or 4, as structural parts of the concave pocket, are also involved in the binding site of PRL. Our results are in agreement with those of Luck et al. (1991) who recently reported that Arg177, localized in the middle of helix 4, is essential for the mitogenic effect of bPRL on Nb2 cells. Similarly, involvement of helix 4 in the PRL binding site could also account for the weak lactogenic and mitogenic properties of 16-kDa prolactin (Clapp et al., 1988), a natural PRL mutant which lacks the C-terminal third of intact PRL, including the whole of helix 4.

In conclusion, the results we have obtained with 24-kDa hPRL appear particularly complementary to our earlier find-

ings that loop 1 is involved in the biological properties of hPRL. The present work brings some contribution to the localization of the global PRL binding site on the folded protein, and strongly suggests other regions of the molecule to be involved in its biological properties. Our results call thus for further analysis of these regions of hPRL and, as for loop 1, each residue constituting the hPRL binding pocket will have to be single-mutated in order to evaluate their involvement in the biological properties of the hormone.

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