Biologically active recombinant human prolactin synthesised and secreted extracellularly in baculovirus expression system

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Summary

The recombinant human prolactin was synthesised as an extracellular protein expressed in baculovirus system. The concentration of prolactin in TC-100 medium was approximately 40 mg/l when the conditions of recombinant virus infection were properly chosen. The human prolactin present in culture medium was stable at 4°C for several months up to one year. The recombinant product was a survival factor for the insect cells. In the presence of prolactin in the medium, the cells did not show any signs of lysis or disruption, which is in agreement with the view of the antiapoptotic action of prolactin. The results of Western blot analysis showed similar ratio of glycosylated/non-glycosylated forms of the recombinant product to the hormone forms present in human physiological (osmiotic) fluids. The recombinant protein was biologically active as determined in mammary gland explant system. The recombinant hormone present in the culture media was shown to induce mRNAs for two milk proteins – β-casein and WAP in mammary explants cultured in the presence of insulin and hydrocortisone. The effect of the hormone was dose-dependant and the largest accumulation of both mRNAs was observed at rec-hPRL concentration of 0.1 μg/ml (approx. 4.3×10^{-9} M). In this respect, the activity of the recombinant human prolactin was equal or even higher than that of bovine pituitary prolactin or human growth hormone.

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1. Introduction

Prolactin is a hormone common in animal world and has highly conserved amino acid sequence (1). The gene encoding human prolactin is composed of five exons and four introns (2,3). Complementary DNA for human prolactin was composed of 914 nucleotides and contains open reading frame coding for pre-hormone of 227 amino acids including a signal peptide of 28 amino acids (4). Circulating human prolactin (hPRL) is a single polypeptide chain of 199 amino acids with three intramolecular disufide bonds (5) and molecular weight of approximately 23 kDa. The hormone is synthesised and secreted by the anterior lobe of the pituitary gland.

Prolactin belongs to protein family which also includes growth hormone (GH) and chorionic somatotropin (placental lactogen, PL; 6). These hormones are evolutionary related and share similar structural, immunological, and biological properties. Prolactin present in blood plasma, amniotic fluid, milk, semen, cervical mucus, and cerebral fluid is involved in more than 85 different biological functions in vertebrates with the most important in the pregnancy: lactation and ovulation [reviewed by Bole-Feysot et al. (7)]. In the mammary gland epithelial cells, expression of milk protein genes is regulated by the co-ordinate action of insulin, glucocorticoids, and prolactin (8). Prolactin levels are higher during ovulation, pregnancy, nursing, and in several other circumstances as for example stress [reviewed by Freeman et al. (9)]. Administration of prolactin also seems potentially useful for the treatment of infections and immunosuppression (10). It was shown that prolactin has a protective effect on pathogen-induced infections in mice (11). Several disorders have been associated with elevated prolactin levels in the organism, as hyperprolactinaemia, which impairs the gonadal function in both sexes. In the production of rec-hPRL, several laboratories developed prokaryotic systems based on *Escherichia coli* synthesising prolactin in the form of inclusion bodies (12-14). Solubilization and renaturation of rec-PRL granules can result in presence of aggregated and immunologically altered product as well as in low expression level (15). More recently, the production of rec-PRL by eukaryotic systems has been shown by Price et al. (16) in murine cells, although carbohydrate structure was significantly different from the natural counterpart. It seems that the proper glycosylation and the yield of rec-hPRL have been proceeded by another eukaryotic system – Chinese-hamster ovary system (17,18). Also baculovirus system using insect cells seems to be very useful in the high yield synthesis of rec-PRL (19,20) in both glycosylated and non-glycosylated forms.

In this paper, we describe the production of secreted rec-hPRL in baculovirus system and show its biological activity in mammary gland explants. The recombinant human prolactin can be applied for pharmacological, medical, and therapeuti-

cal purposes. The main method currently employed for estimation of the level of circulating prolactin is the radioimmunoassay (RIA) which requires availability of the human hormone to use it as an antigen. For this reason, a cheap and efficient method of biologically active hPRL production was developed.

2. Materials and methods

2.1. Cells and reagents

Two insect monolayer cultures have been used: Sf21 for amplification of recombinant virus, and HF cells for production of the protein product. Both cell lines were grown on TC-100 medium supplemented with 10% FBS from Gibco, BRL. Endonucleases, ligase, and Taq polymerase were purchased from Fermentas. All standard procedures: transformation, DNA cleavage and ligation were done according to Sambrook et al. (20). The Bac-N-Blue transfection kit and pMelBac transfer vector for secretion of recombinant proteins were purchased from Invitrogen. The reagents used in the experiments on mammary gland explants were obtained from the following sources: [α-^{32}P]dCTP] (3000 Ci/mmol) from DuPont NEN, medium 199 with Hank`s salts (10×), BME amino acids solution (100×) from Gibco BRL. Hormone preparations were from the following sources: hydrocortisone (4-pregnen-11-α, 17,21-triol-3,20-dione) from Koch-Light; crystalline porcine insulin was a gift from Eli Lilly Co., bovine prolactin was provided by Prof. K. Kochman from the Institute of Animal Physiology and Nutrition, Jabłonna, Poland, and human GH was from Sigma Chemical Co. Rabbit β-casein and whey acidic protein (WAP) cDNAs used as probes were kindly provided by Prof. L.M. Houdebine of INRA, Jouy-en-Josas, France; random labelling kit was from Gibco BRL, England. All other reagents and chemicals were from Sigma and Serva.

2.2. Genetic manipulations

The gene construct was prepared to produce prolactin in baculovirus *in vitro* system. DNA sequence coding for human prolactin between restriction sites BamHI/KpnI was cut out from plasmid pFastPRL (21) and transferred to pMelBac B plasmid, using the same restriction sites. Final recombinant plasmid vector contained a cDNA sequence for hPRL with melittin secretion sequence in front of human prolactin gene under AcMNPV polyhedrin promoter. Recombinant transfer vector pMelPRL was prepared to direct the product to extracellular medium. The scheme of the construct is shown in Figure 1. Recombinant baculovirus MelPRL was obtained by co-tranfection of pMelPRL DNA with Bac-N-Blue AcMNPV DNA according

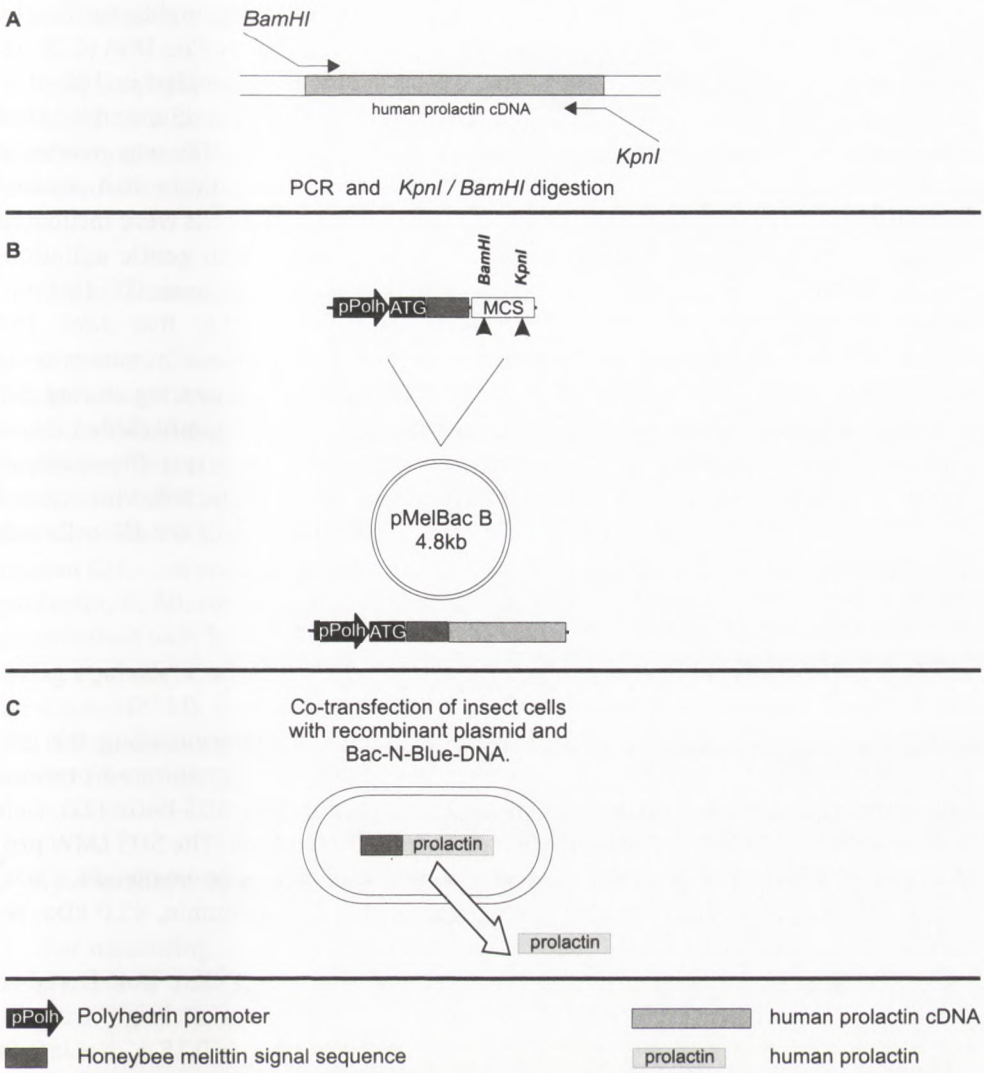

Fig. 1. Schematic representation of prolactin expression in insect cells infected with recombinant baculovirus MelProl. Recombinant human prolatin (rec-hPRL) directed by melittin signal peptide from the honeybee and secreted to the culture medium. The expression under polyhedrin promoter in insect Sf 9 cells has been done.

The cDNA coding for human prolactin was amplified by PCR using primers suitable for trimming gene flanking sequences in *Bam*HI and *Kpn*I restriction sites (A). The cDNAProl was inserted into pFastBac1 donor plasmid which expresses unfused recombinant prolactin and into pFastBacHTa which expresses polyhistidine tagged prolactin (B). The next step was transformation of recombinant plasmids into DH10 Bac™ competent E. coli cells. Recombinant bacmids DNAs (Prol and HisProl) were isolated from selected colonies and then used to transfect Sf21 cells. To direct recombinant prolactin to extracellular medium pMelBac transfer vector was used containing melittin secretion signal in front of inserted human prolactin gene. For generation of recombinant baculovirus (MelProl), the above construct was cotransfected with Bac-N-Blue AcMNPV DNA into Sf9 cells (C). Prolactin expression proceeded in insect cells infected with the recombinant virus.

to modified Invitrogen protocol: (1) MelPRL plasmid DNA (2.5 μg) highly purified by Plasmid Miniprep Plus kit (Biotechnology) was mixed with of Bac-N-Blue DNA (0.25 μg) in 100 μl of TC-100. (2) Lipofectin (Gibco, BRL) 7.0 μl was suspended in 100 μl of TC-100. Both mixtures (1 and 2) were combined and incubated for 45 minutes. After incubation, the transfection mixture, adjusted to 1 ml with TC-100, was overlayed on monolayer culture of Sf21 cells (0.9×10^6) in 30 mm tissue culture dish, washed previously with TC-100 medium without FBS. Subsequently the cells were incubated at room temperature with transfection mixture for 5 hours with gentle agitation. Finally, the transfection mixture was replaced with complete medium, (TC-100 supplemented with 10% FBS) and incubated at 27°C for another five days. The supernatant was than assayed for virus titer in recombinant plaques formation assay according to Invitrogen Manual. Blue recombinant plaques appearing during 5-7 days were carefully examined in inverted microscope to select non-occluded (occ^-) colonies. Clones described as occ^- were screened by PCR to detect the insert of proper length. Clones that did not contain the insert were neglected. Virus clones were amplified in Sf21 cells in the period of 10 days. Infection of the HF cells was done with 1-5 MOI/cell.

2.3. Protein analysis

Proteins from the growth medium were analysed after concentrating the medium with ammonium sulphate at the saturation of 80%; the precipitated proteins were dissolved in 1/10 of original volume and analysed by 12% SDS-PAGE (22). Gels were stained with 0,25% Coomassie Brilliant Blue R-250 (BioRad). The SDS LMW proteins from BioRad were used for molecular weight calibration: lysozyme, 14.4 kDa; trypsin inhibitor, 21.5 kDa; carbonic anhydrase, 31.0 kDa; ovalbumin, 45.0 kDa; serum albumin, 66.2 kDa; phoshorylase B, 97.4 kDa.

For Western blotting experiments PAGE-separated proteins were transferred to Immobilon P membrane (Millipore). After blocking for 4 hours or overnight with the solution of 5% nonfat dry milk in TBST (0.1 M Tris-HCl, pH 7.4; 0,15 M NaCl; 0.1% Tween 20), membranes were incubated for 1 hr with rabbit anti-prolactin polyclonal antibody (Chemicon International Inc., in dilution 1:1000). Membranes were washed 2 times for 10 min with TBST and incubated for 1 hour with a Peroxidase-conjugated Anti-Rabbit IgG (H+L) (Jackson Immuno Research Laboratories, Inc.) diluted 1:5000, washed again with TBST (3×10 min) and developed with DAB substrate Kit (Novo Costra).

Concentration of human prolactin in the media derived from insect cell cultures was assayed by standard RIA procedure (Izo-med., Warsaw, Poland) with radioimmunodiagnostic kit from Immunotech.

2.4. Animals and tissues

New Zealand female rabbits were used in their first pregnancy. Animals were killed by cervical dislocation (in the local abattoir) on day 15 of pregnancy, their mammary glands were excised, cleared from most adjacent muscle, fat and connective tissues and mammary fragments (explants) were prepared, 1 mg weight each.

2.4.1. Cultures of the mammary gland explants

Mammary gland organ cultures were carried out as previously described (23). Explants were incubated for 24 hours at 37°C in an atmosphere of 5% CO_2 in air in medium 199 (Gibco-BRL) supplemented with 5.0 μg/ml insulin and 1.0 μg/ml hydrocortisone. Then, bovine prolactin, human growth hormone or TC-100 media containing rec-hPRL were added and cultures were incubated for additional 24 hours. Bovine prolactin was used at concentrations 0.01, 0.1 or 1.0 μg/ml culture medium; human GH – at concentration of 1 mg/ml. To test the effect of recombinant human prolactin, 6, 60, or 600 μl of TC-100 medium, derived from cultures of insect cells recombined with baculovirus vector, was added to 4 ml of mammary gland cultures, being equivalent of 0.01, 0.1 or 1.0 μg/ml of prolactin (4.3×10^{-10} M, 4.3×10^{-9} M or 4.3×10^{-8} M), respectively. To the control cultures the same amounts of fresh TC-100 medium were added. At the end of the cultures, explants were collected and stored frozen for further analysis.

2.5. Northern blot analysis

For measuring expression of milk protein genes, the concentration of the β-casein and WAP mRNAs was estimated in mammary gland explants by Northern blot analysis. Total RNA was extracted by the guanidinum thiocyanate-phenol-chloroform method of Chomczynski and Sacchi (24). Ten micrograms of RNA were electrophoresed in 1.5% agarose, transferred to Hybond-N nylon membrane (Amersham) and hybridised for 16-20 hours to rabbit β-casein or WAP cDNA probes labelled with [α-^{32}P] dCTP. After hybridisation, filters were washed with 2 × SSPE, 0.1% SDS at room temperature and then with 1 × SSPE and 0.1 × SSPE, 0.1% SDS at 65°C according to filter producer`s instruction. Filters were autoradiographed at -80°C in light-proof cassettes with Hyperfilm (Amersham) placed between two amplifying screens (Dupont, Cronex).

3. Results

Human prolactin was synthesised *in vitro* in insect cells in the eukaryotc baculovirus system. The biologically active peptide product was expressed and released from the cells to the culture medium. The scheme of the genetic construct used for prolactin expression is shown in Figure 1. The cDNA for human prolactin was in-

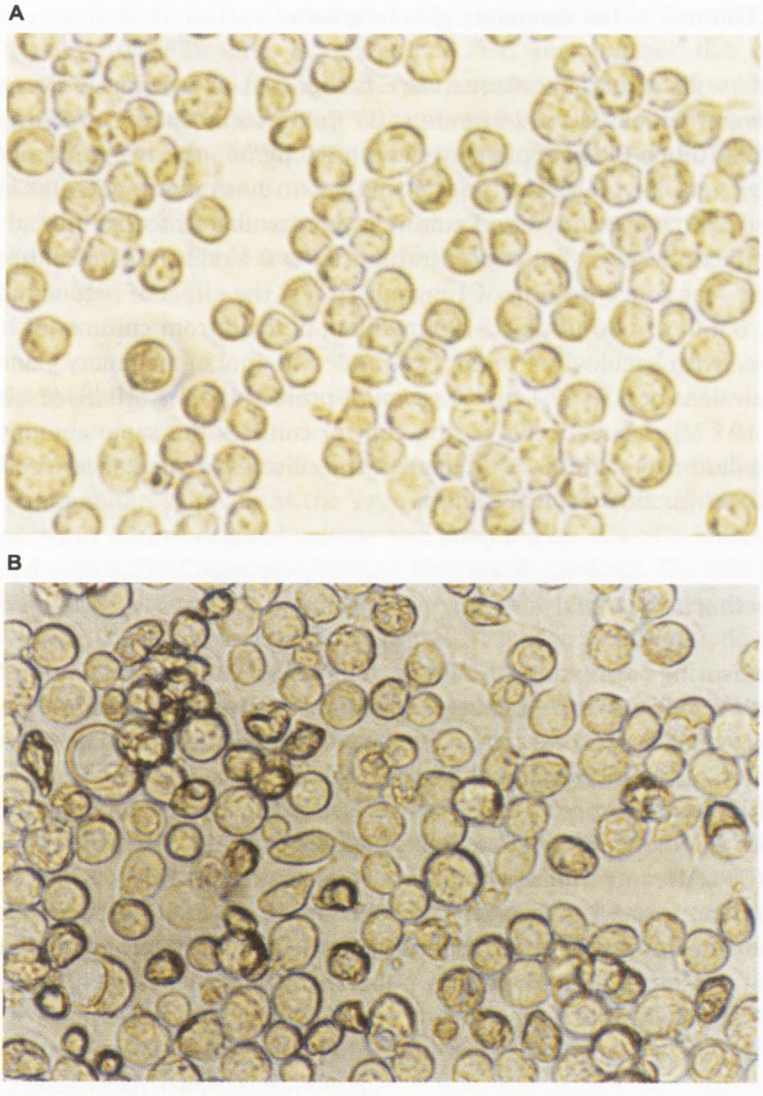

Fig. 2. Non-infected Sf9 insect cells (A), and Sf9 cells 10 days after infection with MelPRL virus (B). No signs of cells lysis or desintegration after viral infection could be seen. This protective effect s characteristic for prolactin presence in the medium. Magnification, 300×.

serted in the multicloning site (MCS) of the pMelBac B plasmid behind melittin secretion sequence. In the insect cells, co-transfection of two DNA molecules was done: recombinant DNA vector and Bac-N-Blue DNA. Recombinant transfer vector pMelPRL containing prolactin sequence proceeded by secretion melittin sequence located under efficient polyherin promoter. In transduction, recombinant plasmid DNA was the source of secretion and PRL sequences introduced subsequently into the virus genome. Such a recombinant virus could infect insect cells and express prolactin synthesised inside the cells to the medium during 5-6 days of incubation.

A protective effect of prolactin present in TC-100 medium on cell survival was observed. In Figure 2, the outlook of Sf21 cells infected at 0.1-0.2 pfu/cell, producing and secreting human prolactin to the medium, ten days after viral infection is shown. Usually, 3-5 days post infection with AcMNPV or its recombinant of the same MOI, insect cells start to brake down and their content leaks to the medium. The protective effect on cell viability is characteristic for prolactin presence in the medium. As seen in Figure 2, uninfected Sf cells (A) and cells ten days after infection (B) did not change; on the contrary, the shape characteristic for dividing cells can be seen under a microscope at 300× magnification.

Electrophoretical analysis of proteins present in the culture medium is shown in Figure 3. Lanes 1 and 2 represent 10 µl and 2 µl of the 10x concentrated medium, respectively; lane 3 is the low molecular weight (LMW) protein standard. Since the cells were grown in TC-100 medium supplemented with 10% FBS, the main protein fraction

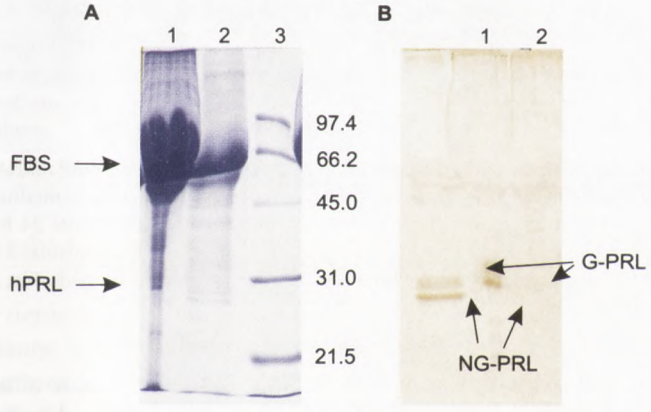

Fig. 3. Polyacrylamide gel electrophoresis (PAGE) and Western blot analysis of proteins present in the TC-100 medium supplemented with 10% FBS, 120 hours post infection with MelPRL virus. The medium was concentrated by 80% saturation with $(NH_4)_2SO_4$. The 12% gels were stained with Coomassie Brilliant Blue (A) or blotted onto PVDF membrane (B) and incubated with polyclonal antibody against hPRL and then to peroxidase-labelled secondary antibody. Identical samples were loaded in parallel on gels A and B. Lane 1 – 10 µl of 10× concentrated medium; lane 2 – 2 µl of 10× concentrated medium; lane 3 – low molecular weight (LMW) protein standards. G-PRL – glycosylated prolactin; NG-PRL – non-glycosylated prolactin.

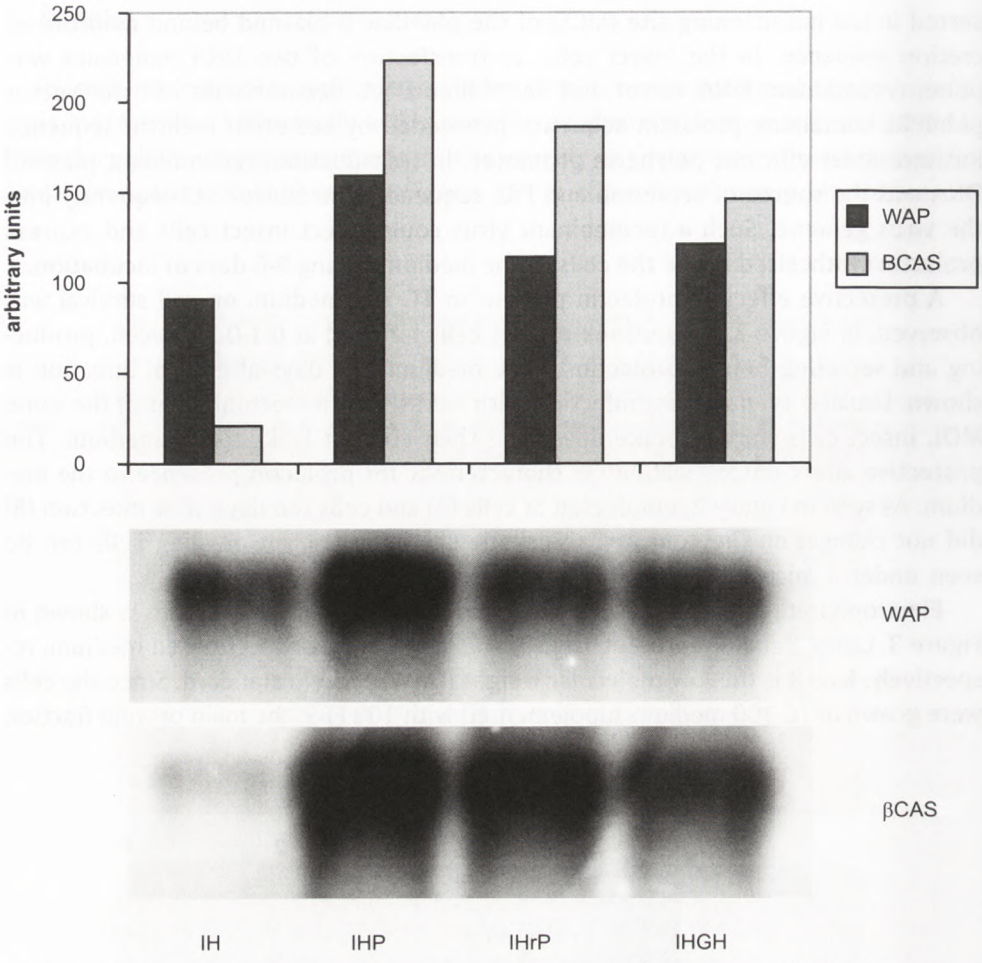

Fig. 4. Northern blot analysis of the accumulation of β-casein mRNA and WAP mRNA in the mammary gland organ cultures. Explants derived from pregnant rabbits were incubated in medium 199 in the presence of insulin (I) and hydrocortisone (H) for 24 hours, and then for additional 24 hours with bovine prolactin (P), rec-hPRL (rP) or human growth hormone (GH), all at the concentration of 1.0 μg/ml medium. Then, total RNA was extracted from mammary tissues, transferred to nylon filters and hybridised to [^{32}P]dCTP labelled β-casein or WAP cDNA probes.

seen on 12% gels stained with Coomassie Brilliant Blue was bovine albumin (Fig. 3A). Nevertheless, prolactin can be also seen as a faint double band, representing glycosylated and non-glycosylated hormone. Figure 3B shows the Western blot analysis of the samples identical to those in Figure 3A. Reaction with rabbit anti-prolactin antibody and with secondary antibody (peroxidase-conjugated antirabbit IgG) visualises a double band of human prolactin. The proportion of two forms of prolactin: glycosylated and non-glycosylated, visualised in Western blotting analysis, was estimated for approximately 1:4. It means that non-glycosylated form is more abundant.

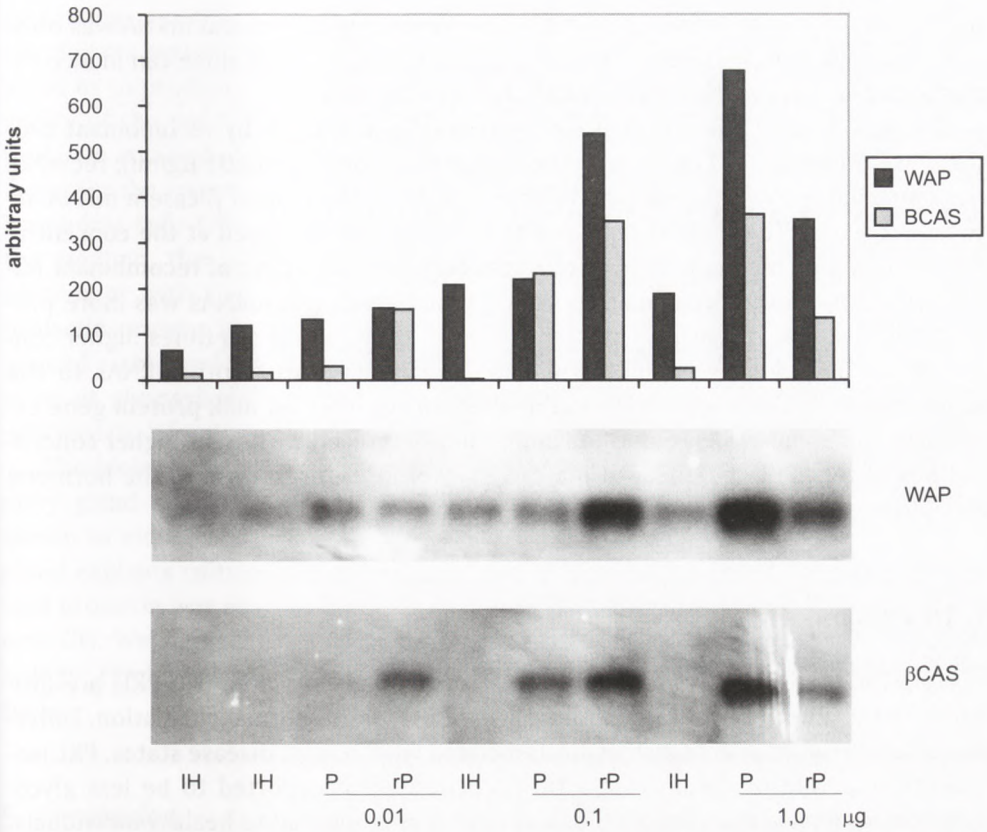

Fig. 5. Dose response of β-casein and WAP mRNAs to bovine pituitary prolactin (P) or to rec-hPRL (rP). Conditions for explant culture are the same as in Figure 4 with the exception that prolactins were added at concentrations ranged from 0.01 to 1.0 mg/ml. IH – control explants cultured with insulin and hydrocortisone, alone.

The biological activity of the human prolactin synthesised by insect cells was assessed by means of its effect on mammary tissues in the organ culture. Mammary gland explants derived from pregnant rabbits were cultured in the presence of insulin and hydrocortisone with or without addition of prolactin. In the mammary explants cultured in the presence of insulin and hydrocortisone, the recombinant human prolactin, equally to bovine pituitary prolactin, induced mRNAs for two milk proteins – β-casein and WAP (Fig. 4). Explants cultured for 24 hrs in the presence of rec-PRL or bovine pituitary prolactin accumulated 10- or 9-times more β-casein mRNA, respectively, then the controls cultured with IH alone. Human growth hormone (GH), the hormone of a known lactogenic activity, stimulated accumulation of β-casein mRNA about 7-fold. The WAP mRNA levels were increased by 1.75-fold, 1.25-fold, and about 1.3-fold in the presence of P, rP and GH, respectively. The smaller effect of prolactins

and GH on the accumulation of WAP mRNA as compared to β-casein mRNA was obviously due to the known phenomena (24,25) that glucocorticoids alone can induce expression of WAP gene, thus increasing the IH control levels.

The results of a dose-response activation of two mRNAs by recombinant prolactin are presented in Figure 5. At the lowest concentration (0.01 μg/ml), rec-hPRL was much more powerful than the bovine, at least in the case of β-casein mRNA accumulation test. The highest response to rec-hPRL was observed at the concentration of 0.1 μg/ml medium. Also, at this concentration the effect of recombinant human prolactin on the accumulation both β-casein and WAP mRNAs was more pronounced then that of bovine pituitary prolactin. However, at ten times higher concentration (1.0 μg/ml), rec-hPRL stimulated accumulation of both mRNAs to the lesser extent. At this concentration, the effect of rec-hPRL on milk protein gene expression was smaller then that of bovine pituitary prolactin. Thus, at higher concentrations of rec-hPRL the effect of plateau or overdose in response to the hormone was observed.

4. Discussion

Two forms of PRL, glycosylated (G-PRL) and non-glycosylated (NG-PRL) are produced in the anterior pituitary. Both forms are present in normal circulation. Differences in PRL ratios or activity may be associated with certain disease states. PRL isolated from serum of rheumatoid arthritis patients was reported to be less glycosylated and to have decreased biological activity as compared to healthy individuals. G- and NG-PRLs have demonstrated different immunological, biological and receptor binding properties.

Several *in vitro* expression systems have been used to synthesize human prolactin. The hormone was synthesised and accumulated in inclusion bodies of bacteria *Escherichia coli* (27). The recombined product was similar to natural human prolactin, except for an additional methionine group at the amino terminal and antibody raised to this product blocked completely antigen-antibody cross-reactivity. The high-level expression of biologically active human prolactin in the baculovirus system was reported by Das et al. (19). The production of recombinant prolactin in this system varied from 20 to 40 mg/l. The hormone secreted to the culture media seemed to be properly glycosylated, and was biologically active with Nb2 cells.

In our laboratory, we also developed an eukaryotic baculovirus system for expression of human prolactin (21). The recombinant protein was synthesized with 6xHis residues attached to N-terminus. The product was thoroughly purified on Co^{++}-affinity column giving a highly purified protein in two forms: glycosylated and non-glycosylated.

The aim of this work was to develop a similar system for more efficient synthesis of human recombinant prolactin suitable for purification procedure avoiding de-

naturating conditions. For this reason, the genetic construct based on the baculovirus system was prepared which, after infection of the insect cell line, enabled us to produce recombinant product secreted to the culture medium. The efficiency of the product expression was high and the concentration of recombinant prolactin attained 40 mg/l medium at optimal conditions. The product was a survival factor for the insect cells, similarly as it has been previously demonstrated with Nb2 lymphoma cells (28). Ten days after viral infection, in the presence of prolactin in the medium, the cells did not show any signs of lysis or disruption, which is in agreement with the view of the antiapoptotic action of prolactin. Interestingly, prolactin present in the medium was characterised by a very high stability. It remained stable and active for several months up to one year, with constant proportions of glycosylated and non-glycosylated forms. The ratio of glycosylated/non-glycosylated forms was estimated approximately for 4:1.

The biological activity of the recombinant prolactin was demonstrated in mammary gland explants. Recombinant human prolactin secreted to the media was shown to induce accumulation of β-casein and WAP mRNAs in the rabbit mammary gland explants cultured *in vitro*. In this respect, the activity of the recombinant human prolactin was equal or even higher than that of bovine pituitary prolactin or human GH. We suggest that the recombinant human prolactin, a product of biotechnology, can be useful for biological, medical as well as for pharmaceutical applications.

5. Conclusion

The recombinant human prolactin can be synthesized as an extracellular protein expressed in baculovirus system. The concentration of prolactin in the culture 100 medium was high and reached 40 mg/l. The human prolactin produced was stable in culture medium for several months, up to one year. The recombinant protein was biologically active. It was shown to induce expression of two milk proteins – β-casein and WAP – in mammary explants. In this respect, the activity of the recombinant human prolactin was equal or even higher than that of bovine pituitary prolactin or human growth hormone. The recombinant prolactin was properly glycosylated; the ratio of glycosylated to non-glycosylated forms was approximately as 4:1. In conclusion, we consider the insect cells and the baculovirus system suitable for production of biologically active human prolactin. We suggest that the recombinant human prolactin can be useful for biological, medical, and pharmaceutical applications.

Abbreviations:

PRL – prolactin; G-PRL – glycosylated prolactin; NG-PRL – non-glycosylated prolactin; hPRL – human prolactin; rec-hPRL – recombinant human prolactin; GH – growth hormone; WAP – whey acidic protein; FBS – foetal bovine serum; M.O.I. – multiplicity of infection; occ$^-$ – non-occluded virus; MelProl – recombinant *Autographa californica* virus with PRL sequence preceded by honeybee secretion sequence.

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Literature

1. Niall H. D., Hogan M. L., Sauer R., Rosenblum I. Y., Greenwood F. C., (1971), Proc. Natl Acad. Sci. USA, 68, 866-870.
2. Cooke N. E., Coit D., Shine J., Baxter J. D., Martial J. A., (1981), J. Biol. Chem., 256, 4007-4016.
3. Truong A. T., Duez C., Belayew A., Renard A., Pictet R., Bell G. I., Martial J. A., (1984), EMBO J., 3, 429-437.
4. Sinha Y. N., (1995), Endocrinol. Rev., 16, 354-369.
5. Shome B., Parlow A. F., (1977), J. Endocrinol. Metab., 45(5), 1112-1115.
6. Horseman N. D., Yu-Lee L. Y., (1994), Endocrinol. Rev., 15, 627-6649.
7. Bole-Feysot Ch., Goffin V., Edery M., Binart N., Kelly P. A., (1998), Endocrinol. Rev., 19, 225-268.
8. Devinoy E., Hubert C., Jolivet G., Thepot D., Clergue N., Desaleux M., Servely J. L., Houdebine L. M., (1988), Reprod. Nutr. Dev., 28, 1145-1164.
9. Freeman M. E., Kanyicska B., Lerant A., Nagy G., (2000), Physiol. Rev., 80, 1523-1631.
10. Richards S. M., Garman R. D., Keyes L., Kavanagh B., McPherson J. M., (1998), Cell. Immunol., 184, 85-91.
11. Meli R., Raso G. M., Bentivoglio C., Nuzzo I., Galdiero M., DiCarlo R., (1996), Immunopharmacol., 34, 1-7.
12. Cunningham B. C., Henner D. J., Wells J. A., (1990), Science, 247, 1461-1465.
13. Gilbert M. S., Lowry P. J., Castro M. G., Woods R. J., Savva D., (1991), Int. J. Biochem., 23, 107-114.
14. Hiraoka Y., Nomata Y., Matsuo K., Tsubata N., Tanabe K., Fukusawa T., (1991), Mol. Cel. Endocrinol., 81, 147-154.
15. Morganti L., Soares C. R. J., Affonso R., Gout P. W., Bartolini P., (1998), Biotech. Appl. Biochem., 27, 63-70.
16. Price A. E., Logvinenko K. B., Higgins E. A., Cole E. S., Richards S. M., (1995), Endocrinology, 136, 4827-4833.
17. Kagawa Y., Takasaki S., Utsumi J., Hosoi K., Shimuzu H., Kochibe N., Kobata A., (1988), J. Biol. Chem., 263, 17508-17515.
18. Soares C. R. J., Morganti L., Miloux B., Lupker J. H., Ferrara P., Bartolini P., (2000), Biotech. Appl. Biochem., 32, 127-135.
19. Das T., Johns P. W., Goffin V., Kelly P., Kelder B., Kopchick J., Buxton K., Mukerji P., (2000), Protein. Exp. Purif., 20, 265-273.
20. Sambrook J., Fritsch E. F., Maniatis T., (1989), *Molecular Cloning: A laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
21. Strokovskaya L., Bartoszewicz Z., Szolajska E., Kikhno I., Solomko A., Michalik J., (2001), Protein. Exp. Purif., 22, 242-248.
22. Laemmli U. K., (1970), Nature, 227, 680-685.

23. Zwierzchowski L., Flechon J., Ollivier-Bosquet, Houdebine L, M., (1987), Biol. Cell., 61, 51-57.
24. Chomczynski P., Sacchi N., (1987), Anal. Biochem., 162, 156-159.
25. Hobbs A. A., Richards D. A., Kessler D. J., Rosen J. M., (1982), J. Biol. Chem., 257, 3598-3605.
26. Puissant C., Houdebine L. M., (1991), Cell. Biol. Int., Reports, 15, 121-129.
27. Paris N., Rentier-Delrue F., Defontaine A., Goffin V., Mercier L., Martial J. A., (1990), Biotech. Appl. Biochem., 12, 436-449.
28. Al-Sakkaf K. A., Mooney L. M., Dobson P. R. M., Brown B. L., (2000), J. Endocrinol., 167, 85-92.