Volume 212, number 2, 220–224 FEB 04439 February 1987

Detection and enzymatic deglycosylation of a glycosylated variant of prolactin in human plasma

Jacques Champier, Bruno Claustrat+, Geneviève Sassolas* and Michel Berger

Laboratoire de Biophysique, UFR Alexis Carrel, rue Guillaume Paradin, F-69372 Lyon, +Service de Radiopharmacie et Radioanalyse and **Centre de Médecine Nucléaire, Hôpital Louis Pradel, F-69394 Lyon Cédex 03, France*

Received 25 November 1986

Immunoperoxidase electrophoresis was applied to the plasma of a patient showing a high level of prolactin (PRL) secreted by a pituitary adenoma. Two PRL monomers were detected with an anti-hPRL antiserum: a major 22 kDa form and a minor 25 kDa form. Concanavalin A-Sepharose 4B chromatography revealed that the 25 kDa form was a glycosylated variant of PRL. Incubation of this variant with endoglycosidase F led to its transformation into the 22 kDa form.

Prolactin; Electrotransfer; Immunoperoxidase electrophoresis; Endoglycosidase; (Human plasma)

1. INTRODUCTION

A glycosylated form of prolactin (GPRL) was isolated from ovine [1] and human pituitary glands [2]. This modified hormone presented a molecular mass near 25 kDa which was slightly higher than that of the major form (22 kDa). In addition, Sinha et al. [3] reported an uncharacterized 25 kDa form of PRL present in human plasma which, taking its molecular mass into account, could correspond to the glycosylated variant. More recently, it was found that the human decidual tissue, in vitro, synthesized and released a glycosylated form of PRL [4]. Using ovine pituitaries, Strickland and Pierce [5] isolated a glycosylated form of PRL which was converted to monomeric PRL by digestion with endoglycosidase H, an enzyme that acts on high-mannose type oligosaccharides.

Correspondence address: J. Champier, Centre de Médecine Nucléaire, Hôpital Louis Pradel, F-69394 Lyon Cedex 03, France

Abbreviations: endo F, endo-β-*N*-acetylglucosaminidase F; endo H, endo-β-*N*-acetylglucosaminidase H; GhPRL, glycosylated human prolactin; PRL, prolactin

Here, we have taken advantage of the extreme sensitivity of a new technique, 'immunoperoxidase electrophoresis' (IPE) (i) to determine whether a glycosylated PRL variant could be observed in human plasma and (ii) to test the ability of specific endoglycosidases to deglycosylate this form.

2. MATERIALS AND METHODS

2.1. *Origin of the plasma*

Plasma was taken from a patient presenting a prolactin-secreting pituitary invasive macroadenoma. The plasma PRL level, measured by a specific radioimmunoassay described in [6], was very elevated ($\cong$30 μg/ml; normal range $<$25 ng/ml).

2.2. *Electrophoresis*

Polyacrylamide gel electrophoresis in the presence of dodecyl sulfate was carried out according to [7] with a 5% acrylamide concentration for the stacking gel and a 12% acrylamide concentration for the separating gel. Plasma was diluted in Laemmli sample buffer to obtain a concentration of 1 mg protein/ml and heated for 5 min in a boiling water bath. 100 μl aliquots were applied in

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/87/$3.50

duplicate to the top of a 130 × 200 × 2 mm gel in a Protean 2 slab cell (Biorad, Richmond, USA). Run time was between 4 and 5 h under constant current conditions (30 mA/gel). At the end of the migration, the gel was cut into two symmetrical parts. One part was stained with Coomassie brilliant blue, destained at room temperature in 7% acetic acid and scanned for absorbance in a Vernon spectrophotodensitometer. The other part was submitted to electrotransfer.

2.3. *Electrotransfer*

The electrophoretic transfer of proteins from the gel to a nitrocellulose (NC) sheet with 0.2 μm pores (Sartorius, Göttingen) was achieved as in [8] using a horizontal electrotransfer apparatus (Biolyon, Dardilly, France). Transfer was conducted at a constant voltage of 30 V for 90 min [9]. The NC sheet was saturated with 3% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 24 h. Protein fixation was then achieved in 4% PBS-buffered formaldehyde for 1 h and unoccupied protein-binding sites on the NC sheet were saturated with 0.05% Tween 20 in PBS for 1 h.

2.4. *Immunoperoxidase assay*

The NC sheet was incubated for 48 h at room temperature with anti-hPRL (prepared in our laboratory) at a dilution of 1:500 in the presence of non-immune sheep serum. The anti-PRL antibodies, linked to the immunoreactive sites of PRL, were demonstrated by the immunoperoxidase technique [10] using the peroxidase-antiperoxidase (PAP) reagent (PAP reagents, kit ref.47431, Biolyon). These reagents were designed for the visualization of specific antigens on histological sections but the recommended procedure was unchanged. Peroxidase was then revealed for 10 min in a solution of 3,3′-diaminobenzidine/dimethylformamide (0.5 mg/ml) and 0.01% (v/v) H_2O_2. A brown-colored deposit developed at the immunoreactive sites.

2.5. *Affinity chromatography*

1 ml plasma samples were placed on a 2 ml column of concanavalin A-Sepharose 4B (Pharmacia, Uppsala). The column was rinsed with 20 ml PBS, then eluted with 20 ml of 1 M methyl-α-D-glucopyranoside in PBS buffer. Elution was carried out at 0.2 ml/min and fractions of 2 ml were collected. The samples were lyophilized, radioimmunoassayed for PRL and subjected to IPE as described above.

2.6. *Deglycosylation experiments*

0.1 mg protein of glycosylated material (bound material eluted with glucopyranoside) was incubated for 24 h at 37°C in potassium phosphate buffer (pH 6.5) with 0.2 U endo H or 0.5 U endo F. Endo H and endo F were obtained from Boehringer (Mannheim). Samples incubated with or without endo H or endo F were subjected to IPE as described above.

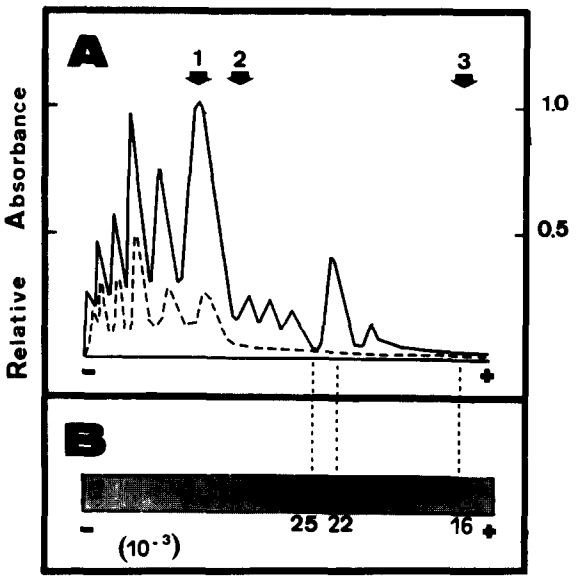

Fig.1. Electrophoresis, electrotransfer and immunoperoxidase revelation of the variants of PRL in human plasma. (A) Demonstration of the efficiency of the electrotransfer procedure. Plasma was loaded in two lanes of the slab. At the end of electrophoresis, one lane was stained and scanned (continuous line), the other submitted to electrotransfer and the remaining material stained and scanned (dashed line). After a 90 min transfer at 30 V, no proteins of <60 kDa remained in the gel and a transfer yield of 90% was obtained for human serum albumin (67 kDa). Arrowheads indicate positions of the marker proteins [1, bovine serum albumin (67 kDa); 2, egg albumin (45 kDa); 3, lysozyme (14.4 kDa)]. (B) Immunoperoxidase pattern of the variants of PRL. Dotted vertical lines indicate the position of PRL variants among plasma proteins. The PRL variants are not visible with Coomassie staining on account of their low plasma concentration.

3. RESULTS

Fig.1A shows the electrophoretic mobilities of plasma proteins and demonstrates the efficiency of this new electrotransfer technique. By immunoperoxidase revelation of the corresponding NC sheet, anti-hPRL allowed the immunostaining of two protein bands: a predominant band corresponding to a 22 kDa form and a minor band corresponding to a 25 kDa form (fig.1B). A 16 kDa form was also found which could correspond to the previously reported cleaved variant, isolated from normal and adenomatous pituitary tissue and from the plasma of pregnant women [11]. Another minor 45 kDa form was sometimes observed but storage of the plasma at −20°C led to the total (or almost total) disappearance of this unstable form (unpublished).

To determine whether these variants were glycosylated, plasma was filtered over concanavalin A-Sepharose 4B and PRL immunoreactivity was measured in the elution volume. 21.0 ± 4.4% (n = 3) of the prolactin immunoreactivity was retained on the column and eluted in a second step with 1 M methyl-α-D-glucopyranoside. Unretained (non-glycosylated material) and retained (glycosylated material) fractions were subjected to IPE. The 22 kDa band corresponded to non-glycosylated PRL (fig.2, lane B) and the 25 and 16 kDa bands to GPRL (fig.2, lane C). When the glycosylated material was submitted to electrophoresis under nondissociating conditions (sample buffer without 2-mercaptoethanol, absence of heating before the run), the 25 kDa form was not detected and was replaced by a larger variant of approx. 60 kDa (fig.2, lane D).

To elucidate the structure of this glycoconjugate, attempts were made to remove the carbohydrate chains of PRL by incubation with specific endoglycosidases. In our hands, endoglycosidase H failed to modify the electrophoretic mobility of the concanavalin

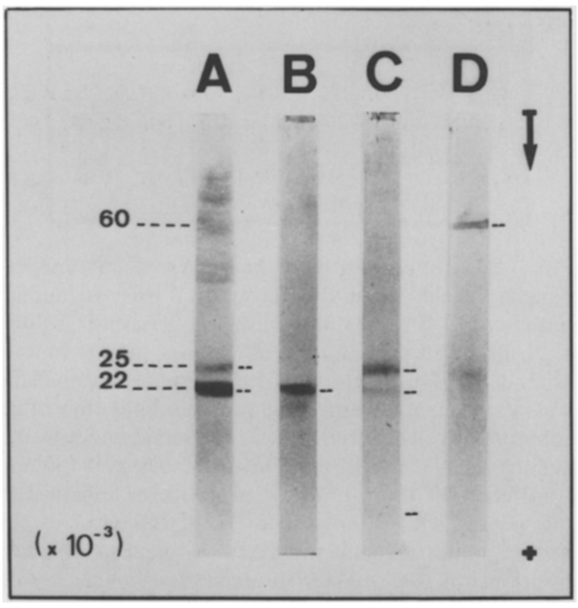

Fig.2. Revelation, using IPE, of PRL variants of human plasma (A), concanavalin A-unretained human plasma (B), concanavalin A-retained human plasma (C) and concanavalin A-retained human plasma after electrophoresis under non-dissociating conditions (D).

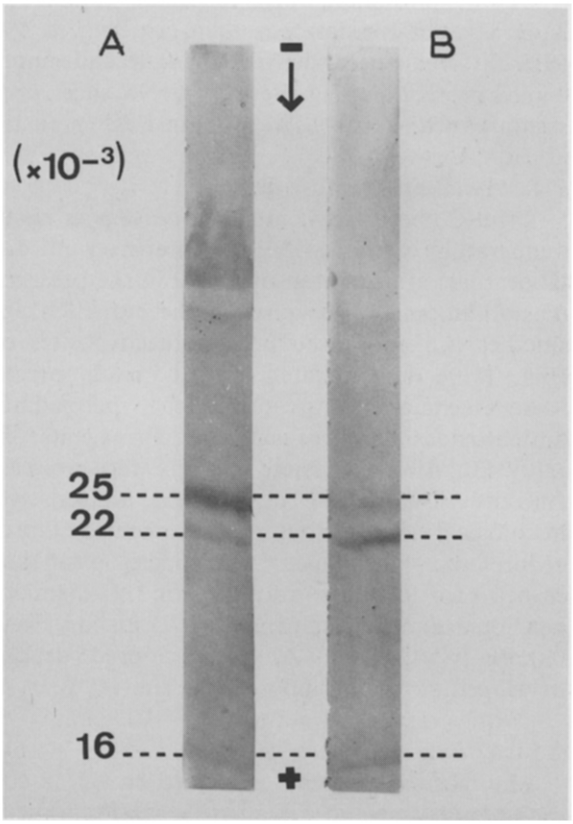

Fig.3. Attempts to deglycosylate GPRL. Concanavalin A-retained human plasma was incubated without (A) or with (B) endo F (0.5 U/0.1 mg protein) for 24 h at 37°C and subjected to IPE.

A-retained PRL (not shown). In contrast, the 25 kDa form was almost completely changed into the 22 kDa form after incubation with endoglycosidase F (fig.3). The electrophoretic mobility of the 16 kDa form was apparently not modified.

4. DISCUSSION

The IPE technique decsribed here is characterized by its extreme sensitivity. This technique was able to detect hPRL and an additional modified variant, without immunoprecipitation before electrophoresis, from the equivalent of 1 μl of our human plasma presenting a very high level of PRL. The apparent molecular mass of this variant is 25 kDa and it could correspond to the uncharacterized PRL variant detected in human plasma [3].

Affinity chromatography experiments indicate that this variant is a glycosylated form of the hormone. To some extent, this glycosylated hormone might be the circulating form of the 29 kDa component which has been detected in crude human pituitary homogenates [12]. Furthermore, a similar glycosylated variant (25 kDa) was recently found in human amniotic fluid between weeks 32 and 40 of normal pregnancy [4]. Shoupe et al. [13] observed only one 60 kDa hPRL-like substance in human plasma of pregnant patients that bound to concanavalin A. This variant ranged from 10 to 30% of the total immunoassayable PRL. These authors isolated this glycosylated form by molecular sieve chromatography on Sephadex G-100 using a nondissociating buffer. Now, it is noteworthy that the electrophoresis of our glycosylated material under nondissociating conditions elicited such a 60 kDa band. This form might represent an aggregate of smaller molecules rather than the true circulating form of the glycosylated hormone in the plasma. Lastly, our results provide strong evidence for the existence of a cleaved glycosylated form ($M_r \cong 16000$) of PRL in human plasma. This form was not shown among nonglycosylated material.

In contrast with a previous report concerning ovine PRL [5], endo H failed to deglycosylate GhPRL in our experiments. Nevertheless, GhPRL was converted to the major form by digestion with endo F. As previously shown, endo F appears very similar in specificity to endo H [14], both hydrolyse *N*-glycans of the high-mannose type [14,15]. The only discrepancy is that endo F cleaves the biantennary chain of an IgM glycopeptide [14]. It may thus be inferred that the added carbohydrate is a high-mannose biantennary oligosaccharide.

As an overall conclusion, using IPE, we have been able to demonstrate the presence of a glycosylated variant of PRL in human plasma, the deglycosylation of which can be achieved by incubation with endo F. If the biological role of this secreted variant is still unknown at present, the multifunctional nature of the PRL, ranging from growth promotion to inhibition of ovulation, could partly result from the existence of different forms of the hormone.

ACKNOWLEDGEMENTS

The authors wish to thank Viviane Thivolle for the preparation of the manuscript and Drs C. Caudie and P. Broquet for helpful discussions.

REFERENCES

[1] Lewis, U.J., Singh, R.N.P., Lewis, L.J., Seavey, B.K. and Sinha, Y.N. (1984) Proc. Natl. Acad. Sci. USA 81, 385–389.
[2] Lewis, U.J., Singh, R.N.P., Sinha, Y.N. and Vanderlaan, W.P. (1985) Endocrinology 116, 359–363.
[3] Sinha, Y.N., Gilligan, T.A. and Lee, D.W. (1984) J. Clin. Endocrinol. Metab. 58, 752–754.
[4] Lee, D.W. and Markoff, E. (1986) J. Clin. Endocrinol. Metab. 62, 990–994.
[5] Strickland, T.W. and Pierce, J.G. (1985) Endocrinology 116, 1295–1298.
[6] Trouillas, J., Girod, C., Sassolas, G., Claustrat, B., Lheritier, M., Dubois, M.P. and Goutelle, A. (1981) J. Pathol. 135, 315–336.
[7] Laemmli, U.K. (1970) Nature 227, 680–685.
[8] Svoboda, M., Meuris, S., Robyn, C. and Christophe, J. (1985) Anal. Biochem. 151, 16–23.
[9] Caudie, C., Plan, E., Najioullah, F., Abouel-Enein, I., Mailfert, G. and Quincy, C. (1986) Electrophorèse Bidimensionnelle, pp.77–84, Press. Univ. Nancy.
[10] Meuris, S., Svoboda, M., Christophe, J. and Robyn, C. (1984) Anal. Biochem. 143, 163–169.

[11] Sinha, Y.N., Gilligan, T.A., Lee, D.W., Hollingsworth, D. and Markoff, E. (1985) J. Clin. Endocrinol. Metab. 60, 239–243.
[12] Meuris, S., Svoboda, M., Vilamala, M., Christophe, J. and Robyn, C. (1983) FEBS Lett. 154, 111–115.
[13] Shoupe, D., Montz, F.J., Kletzky, O.A. and Dizerega, G.S. (1983) Am. J. Obstet. Gynecol. 147, 482–487.
[14] Tarentino, A.L., Gomez, C.M. and Plummer, T.H. (1985) Biochemistry 24, 4665–4671.
[15] Trimble, R.B. and Maley, F. (1984) Anal. Biochem. 141, 515–522.