Involvement of lysine residues in the binding of ovine prolactin and human growth hormone to lactogenic receptors

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The lactogenic activity (L.A.) of oPRL and hGH derivatives obtained by chemical modifications of lysine residues was studied by radioreceptor assay. Control treatment with borohydride had a slight effect on the L.A. of hGH but drastically reduced the oPRL activity; this latter was preserved in the presence of iodoacetamide. Methylation, ethylation, guanidination and acetimidination affected the L.A. of both hormones as a function of the degree of modification. The structure-binding relationships to the lactogenic receptors are discussed, suggesting that the lysine or arginine residues in homologous positions 42, 51, 73, 128, 146 of oPRL and 47, 50, 73, 128, 147 of hGH might be particularly involved.

Prolactin *Growth hormone* *Lysine residue* *Receptor* *Structure*

1. INTRODUCTION

Ovine prolactin (oPRL) and human growth hormone (hGH) belong to 2 families of polypeptides with closely related structures probably evolved from a common ancestral short peptide [1]. A considerable number of amino acids has been preserved during evolution. A hypothetical molecular model of an archetype of the superfamily comprising prolactins, growth hormones and placental lactogens has even been proposed [2]. As regards their genetic origin, we know that hGH and oPRL are encoded by genes located on different chromosomes [3]. During phylogenetic evolution some of these molecules, such as hGH or ovine chorionic somatomammotropin (oCS), preserved both their lactogenic and growth-promoting activities. Other molecules, such as prolactin or human placental lactogen, only exhibit lactogenic activity. Moreover some others, such as bovine GH (bGH), only act as growth-promoting hormones. The close structural relationship between proteins exhibiting a lactogenic activity suggests that certain sequences and a common topographical arrangement are involved in the interaction of these hormones with their specific receptors and in the induction of the activity.

We have previously investigated whether lysine residues were involved in the binding of the oCS molecule to lactogenic and somatotropic receptors [4]. Here, we studied the effects of some chemical modifications of lysine residues of 2 lactogenic hormones (oPRL and hGH) on the binding to lactogenic receptors. The examined hormones have 9 lysine residues, but some of these residues are not located in the same position in the amino acid sequence. Thus, comparison of the results obtained with these 2 proteins may lead to a better understanding of the contribution of lysine residues to their biological activity. Only chemical modifications preserving the basicity of the group (methylation, ethylation, guanidination and acetimidination) were performed, since the abolition of positive charges can cause secondary effects

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on the conformation of the protein. The biological activity was investigated using a radioligand receptor assay with rabbit mammary homogenates.

2. MATERIALS AND METHODS

2.1. *Chemical modifications*

Ovine prolactin was a purified preparation kindly provided by NIH (NIH PS7: 24 IU/mg). Human growth hormone (1 IU/mg) was a gift from Drs Dray and Groh (Institute Pasteur, Paris).

2.1.1. Reductive alkylation

Reductive methylation and ethylation were carried out by addition of sodium borohydride and formaldehyde or acetaldehyde to the hormones (0.5–1.0 mg) dissolved in borate buffer (pH 9) as in [5]. The solutions were dialyzed against a pyridine solution (1%) and freeze-dried. Determinations of lysine, and methyl- and ethyllysine were made using a Technicon autoanalyzer and elution gradients containing isopropanol [5,6]. A blank for biological assays was prepared by treating hormone with borohydride and borate buffer (pH 9, no added aldehyde). In the case of ovine prolactin whose disulfide bridges are particularly labile to the reductive action of borohydride (BH_4^-), the reductive alkylation was performed at a lower concentration of BH_4^- (20 mM) for a shorter period (30 min) and in the presence of iodoacetamide (40 mM) to block the SH groups, avoiding the disordered reconstitution of disulfide bonds.

2.1.2. Guanidination

This reaction was performed using 0.3 M *O*-methylisourea sulfate (Aldrich, France) at pH 10.3 and 5°C (0.7 mg hormone/0.15 ml) for 24 h. At the end of the reaction, the solutions were dialyzed against pyridine solutions. After 1 day of reaction, the solution became slightly opalescent. In both cases, the solutions were centrifuged and the precipitate discarded. The degree of guanidination was measured by amino acid analysis. A control was prepared by treatment of the hormone at pH 10.4 for 1 day at 5°C.

2.1.3. Acetimidination

Prolactin (0.7 mg/0.5 ml) was treated with 1 M ethylacetimidate hydrochloride (Aldrich) for 24 h at pH 10.3 and 5°C. hGH was treated under the same conditions but at a much lower concentration of reagent (0.01 M) to obtain a small degree of chemical modification. Determination of the ϵ-acetimidyllysine was performed by amino acid analysis as in [7].

2.2. *Radioreceptor assay of lactogenic activity*

This activity was measured as in [8]. Mammary gland membranes were obtained from rabbits treated on day 10 of lactation with 2-α-bromocryptine (CB 154, Sandoz, 2 mg twice daily for 2 days) to desaturate their receptors. The standard curve was established by incubation of membrane receptors, radioiodinated prolactin and different concentrations of unlabelled ovine prolactin (NIH PS7, 24 IU/mg) for 5 h at 21°C. Lactogenic activity of modified hormones was determined by adding these hormone derivatives to the incubation medium instead of the unlabelled prolactin. The specificity of the assay was checked: only hormones with lactogenic activity in the rabbit (prolactins of different species, placental lactogens and human growth hormone) are able to compete with ovine prolactin at the rabbit mammary receptor sites. The rabbit receptor exhibits less strict specificity than the ovine receptor.

3. RESULTS AND DISCUSSION

The percentage of modified lysine residues determined by amino acid analysis is reported in table 1. In both methyl- and ethyllysine, the positive charge is preserved almost without change of the p*K* or of its position in relation with the peptidic backbone. However, the ethyl chain is longer than the methyl one and therefore greater steric hindrance may occur in binding to the receptors. Nevertheless, the degree of ethylation was lower in the proteins studied and the more disturbing action of the ethyl group can be compensated by a lower degree of modification.

The degree of guanidination was similar to that of methylation. The degree of acetimidination was much higher in the case of ovine prolactin because of the more drastic reaction conditions.

Reductive alkylation was performed in the presence of BH_4^- which can affect some very labile disulfide bonds. Ovine prolactin subjected to BH_4^- at pH 9 lost about 70% of its biological activity

Table 1

Degree of modification of lysine residues and binding capacity of modified oPRL and hGH to lactogenic receptors

Modified hormones	BH_4^-, pH 9 (control)		Methylated		Ethylated		pH 10 (control)		Guanidinated		Acetimidinated	
	D.M.	L.A.	D.M.	L.A.	D.M.	L.A.	D.M.	L.A.	D.M.	L.A.	D.M.	L.A.
oPRL	0	90[a]	80	24	45	73	0	100	66	21	80	20
hGH	0	90	74	47	52	75	0	100	52	67	10–20	100

[a] Reductive alkylation was performed in the presence of iodoacetamide

D.M., degree of chemical modification of the lysine residues expressed as a percentage; L.A., lactogenic activity expressed as a percentage of the binding activity of native hormone calculated by comparison of the hormone concentration needed for 50% inhibition of specific binding

(fig.1). When iodoacetamide was added immediately after the reduction treatment, the loss of activity was still considerable. However, when using the treatment in the presence of iodoacetamide, most of the activity of oPRL was preserved (fig.1 and table 1). Amino acid analysis showed in the latter case the presence of carboxymethylcysteine (0.5 residue per molecule) suggesting that reconstitution of disulfide bonds was prevented by blocking the -SH groups. A decrease in the lactogenic activity of oCS was also observed after treatment with BH_4^- without iodoacetamide [4]

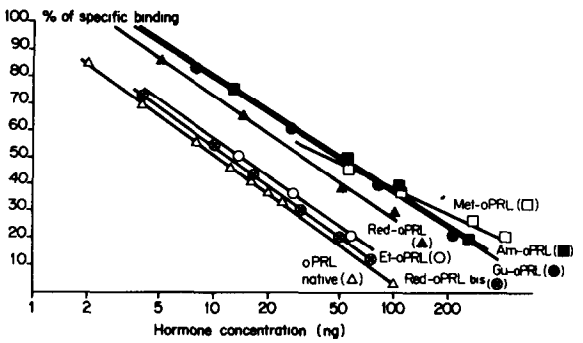

Fig.1. Specific binding of native and modified oPRL in a radioreceptor assay for lactogenic activity with lactating rabbit mammary membranes. (△—△) Native oPRL; (▲—▲) Red-oPRL: reduced oPRL (treated with BH_4^- without iodoacetamide); (⊗—⊗) Red-oPRL bis: reduced oPRL (treated with BH_4^- in the presence of iodoacetamide); (□—□) Met-oPRL: methylated oPRL; (○—○) Et-oPRL: ethylated oPRL; (●—●) Gu-oPRL: guanidinated oPRL; (■—■) Am-oPRL: acetimidylated oPRL.

which probably could be avoided by adding this reagent.

As seen in table 1 and figs 2,3, ethylation only slightly affected the lactogenic activity of both hormones (oPRL, hGH) but the degree of modification was smaller than in the case of methylation. The loss of activity was significant in the methylated hormones. Decreased activity was also observed in the case of the guanidyl derivatives. The fact that guanidyl oPRL exhibited a much lower lactogenic activity could be explained by the modification of some lysine residues corresponding to arginine residues in the hGH molecule (such as Lys 146 and Lys 193, see fig.3) where the sequences of oPRL and hGH are reproduced as reported in [2,9].

The results reported above suggest that some lysine residues of hormone molecules are involved in the interaction between oPRL or hGH and their lactogenic receptors even though the repercussion of their chemical modification on biological activity was less drastic than in the case of somatotropins [10]. Some lysine residues were particularly well preserved during evolution: Lys 128 and 187 are common to all prolactins (in rat, pig, sheep, cattle and man), hCS, the only placental lactogen whose primary sequence was reported and all known growth hormones (in rat, pig, horse, sheep, cattle and man) (fig.3) [2,3,9,11–17]. Lys 42 and 51 of oPRL could correspond approximately in hGH sequence to the positions of Lys 47 and 50, which could occupy a similar place in the steric conformation of the molecule when the gaps are excluded. Lys 42 and 51 of oPRL were very conser-

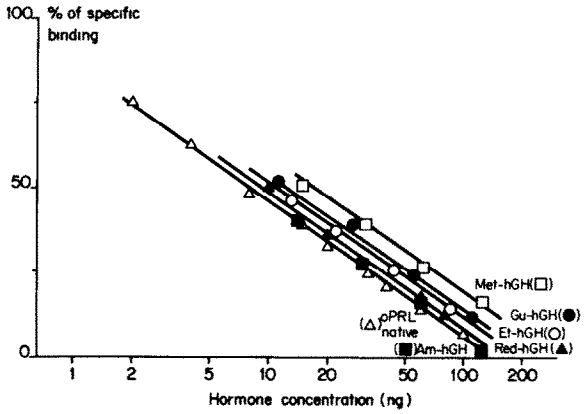

Fig.2. Specific binding of native and modified hGH in a radioreceptor assay for lactogenic activity with lactating rabbit mammary membranes. (Δ—Δ) Native hGH; (▲—▲) Red-hGH: reduced hGH (treated with BH_4^- without iodoacetamide); (□—□)Met-hGH: methylated hGH; (○—○) Et-hGH: ethylated hGH; (●—●) Gu-hGH: guanidinated hGH; (■—■) Am-hGH: acetimidylated hGH.

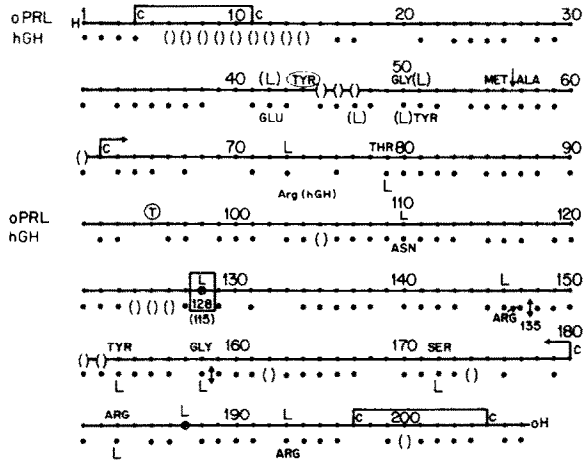

Fig.3. Comparison of oPRL and hGH primary sequences oPRL and hGH. (—●—) Amino acid; ( ) gap; L, lysine; (●) amino acid in the hGH sequence different from oPRL sequence; (⊙) position occupied by a lysine residue in all known prolactins, growth hormones and human placental lactogen; (↓) enzymatic rupture of hGH by trypsin; (↕) enzymatic rupture of oPRL by plasmin.

vative during evolution. Although rat prolactin showed a very high rate of evolution (58.7 accepted point mutations/100 residues per 10^8 years) in comparison with other prolactin molecules [18], residues 42 and 51 of rat PRL are arginine. Residue 50 of hGH was preserved in hCS and all known growth hormones but Lys 47 of hGH and hCS was substituted by a glutamic acid in bovine, ovine, equine, rat GH. These latter hormones are not lactogenic.

Other prolactin lysines (73, 146, 193) are substituted by arginines in hGH (positions 73, 147 and 193 in fig.3). However, Arg 183 of oPRL is replaced by Lys in hGH. Thus these conservative amino acid residues could play a role in the binding to lactogenic receptors. At the present time, we can add that enzymatic rupture by plasmin of the peptide bond between Met 53 and Ala 54 of oPRL (positions 56 and 57 in our scheme because of gaps, fig.3) destroys the biological activity [19]. These results suggest that the N-terminal segment of the oPRL molecule has a significance for lactogenic activity and that the lysines located in this part of the sequence should be involved in the interaction with the receptors. Partial lactogenic activity was also exhibited by fragments 1–134 of hGH [20,21] corresponding to 1–147 in fig.3. In contrast, lactogenic activity was preserved after selective removal with trypsin of residues 135–145 of hGH suggesting that Lys 153 and 158 are not implicated in the function [22]. Other lysine residues were not preserved during evolution and not substituted by a basic amino acid residue, i.e. lysines in position 110 of the oPRL molecule or in position 172 of the hGH molecule (fig.3). To summarize, amino acid residues of oPRL in positions 42, 51, 73, 128, 146 and 47, 50, 73, 128, 147 of hGH might be particularly involved in the binding to lactogenic receptors. Further studies will be required to locate and define precisely the contribution of these lysines to the lactogenic activity.

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