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Relationships between structure and function of lactogenic hormones

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Summary. Lactogenic activity of several hormone derivatives obtained by chemical modifications of lysine residues was studied by radioreceptor assay.

The relationships between structure and binding to lactogenic receptors are discussed taking into account lysine residue positions liable to be involved in the location of lactogenic function.

Introduction.

Placental hormones (PL) or chorionic somatomammotropins (CS) and hypophyseal hormones such as prolactins (PRL) or some growth hormones (GH) exhibit lactogenic activity in radioreceptor assay (RRA) ; they belong to two families of polypeptides with closely related structures probably resulting from a common ancestral short peptide (Niall *et al.*, 1971).

In some ruminants, PL hormones also exhibit a growth hormone activity (Chan *et al.*, 1976 ; Martal and Djiane, 1977). In human female, hCS is unable to bind to growth hormone receptors (Tsushima and Friesen, 1973), while hGH is endowed with both somatotropic and lactogenic action. Besides, amino-acid sequences of hGH, hCS and oPRL are well known (Niall *et al.*, 1971 ; Li *et al.*, 1971). Conversely, the oCS primary structure has not yet been elucidated, though the physicochemical characterization has been performed by Handwerger *et al.* (1974), Martal and Djiane (1975), Chan *et al.* (1976). For all these reasons, these four molecules are very interesting compounds for studying relationships between chemical structure and biological activity.

In the present work, we studied the effects of some chemical changes in lysine residues of these four hormones upon the binding capacity to lactogenic receptors. Only chemical changes preserving the basicity of the group (methylation, ethylation, guanidination and acetiminidation) were achieved, preventing proteins from secondary effects on their conformation.

Afterwards, a comparison was made between the already known primary structures of these hormones in relationship to their lactogenic activity.

Material and methods.

Hormones.

oCS was purified in our laboratory and was not subjected to any contamination from either GH or PRL as reported by Martal (1978).

Global aminoacid composition was established by means of a Technicon autoanalyser. Lysine content was 14 residues for a MW of 22 000 daltons.

Ovine prolactin was a purified preparation kindly provided by NIH (PS-7).

hGH was a gift from Drs F. Dray and F. Groh (Institut Pasteur, Paris).

hCS was provided by USB Corporation.

Chemical modifications.

Four chemical modifications were performed. Methylation was accompanied by a slight change in the pK. Ethylation introduced a longer alkyl chain. Guanidination and acetimidation modified the pK of the protein and the distance between the positive charge of the protein and the polypeptide backbone.

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 described by Means and Feeney (1968). The solutions were dialyzed against a pyridine solution (1 %) and freeze-dried. Determinations of lysine, methyl- and ethyllysine were made using a Technicon autoanalyser and elution gradients containing isopropanol (Means and Feeney, 1968 ; de la Llosa *et al.*, 1974). 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 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.

Guanidination. — This reaction was performed using 0.3 M 0-methyl-isourea 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 one day of reaction, some of the solutions became slightly opalescent. In both cases, the solutions were centrifuged and the precipitate discarded. The degree of guanidination was measured by aminoacid analysis. A control was prepared by treatment of the hormone at pH 10.4 for one day at 5 °C.

Acetimidation. — Prolactin (0.7 mg/0.5 ml) was treated by 1 M ethylacetimidate hydrochloride (Aldrich) for 24 h at pH 10.3 and 5 °C. hGH was treated in 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 -acetimidyl-lysine was performed by aminoacid analysis (Plapp and Kim, 1974).

Radioreceptor assay of lactogenic activity. — This activity was measured as described by Martal and Djiane (1975).

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 on the rabbit mammary receptor sites. The rabbit receptor exhibits less strict specificity than the ovine receptor.

Results and discussion.

Table 1 lists the relative potencies of modified hormones compared to the native hormone (in %).

A decrease in the lactogenic activity of oCS was observed after treatment with only $(\text{BH}_4)^-$ as shown by Chêne *et al.* (1984). This might be due to the disturbance in the conformation caused by partial reduction of disulfide bridges.

This effect could be avoided by adding iodoacetamide reagent in the reductive step as it was done for oPRL (fig. 1, Table 1).

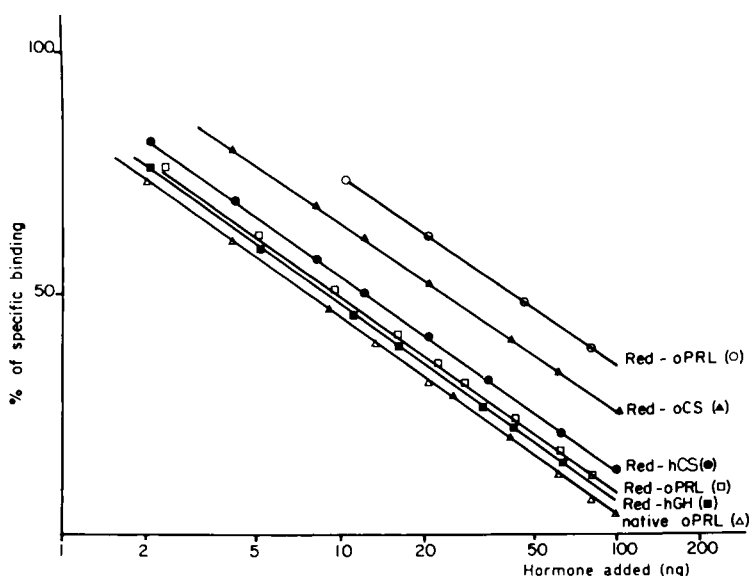


FIG. 1. — Specific binding of reduced hormones in a radioreceptor assay.

- — ○ reduced oPRL (treated by BH_4^- without iodoacetamide)
- — □ reduced oPRL (treated by BH_4^- in the presence of iodoacetamide)

TABLE 1

Compared analysis of the influence of chemical modifications of lactogenic hormones upon their biological activity.

Modified hormones	Reduction	Methylation	Ethylation	Control at pH 10	Guanidination	Acetimination
oPRL (9L)*	90 % (1) (OL)**	24 % (7L)	73 % (4L)	100 % (OL)	21 % (6L)	20 % (6L)
hCS (9L)*	83 % (2) (OL)	70 % (6L)	80 % (2L)	100 % (OL)	0 % (7L)	27 % (4-5L)
hGH (9L)*	90 % (2) (OL)	47 % (7L)	75 % (5L)	100 % (OL)	67 % (5L)	100 % (1-2L)
oCS (14L)*	45.5 % (2) (OL)	38 % (10L)	10 % (8L)	100 % (OL)	1 % (11L)	29 % (3L)

* Number of total lysine residues ; ** Number of modified lysine residues ; (1) Protected disulfide bonds ; (2) No protected disulfide bonds.

In figure 2, methylation seemed very perturbing especially for oPRL. In this reaction, hCS preserved a good activity whereas hGH lost 50 % of its activity.

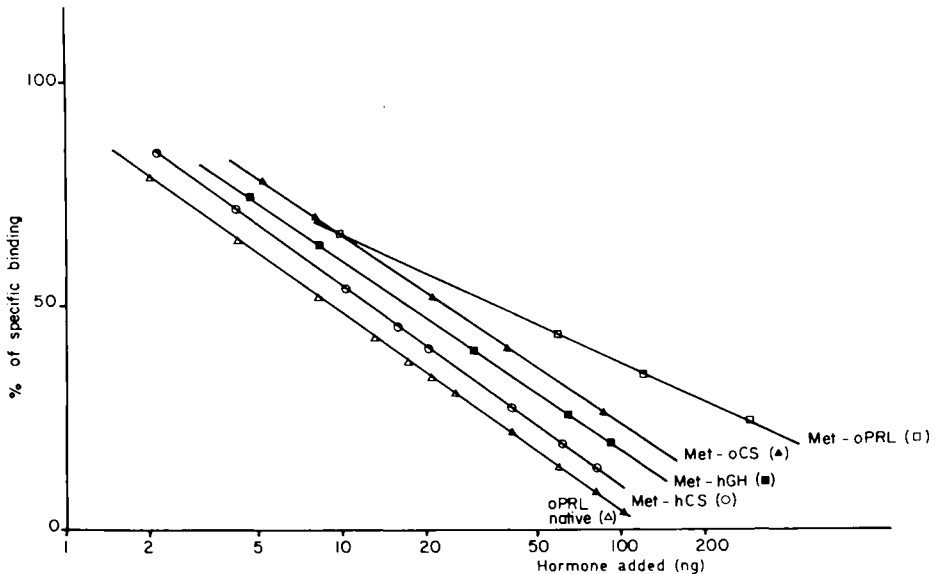


FIG. 2. — Specific binding of methylated hormones in a radioreceptor assay.

In figure 3, ethylation only slightly affected the binding activity of oPRL, hCS, hGH, but the degree of modification was smaller than that due to methylation. Only oCS exhibited a markedly reduced capacity.

A 5-day treatment of the four hormones at pH 10.4 and at 4 °C (necessary for guanidination and acetimination) did not apparently affect their biological activity.

Lactogenic activity of guanidylated compounds was largely depressed (except for guanidyl-hGH), those of hCS and oCS were completely abolished (see fig. 4, table 1).

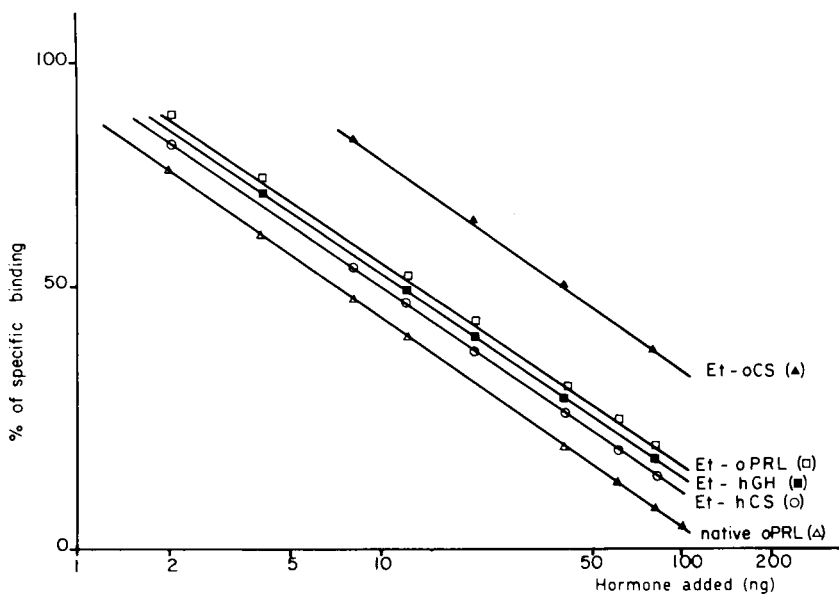


FIG. 3. — Specific binding of ethylated hormones in a radioreceptor assay.

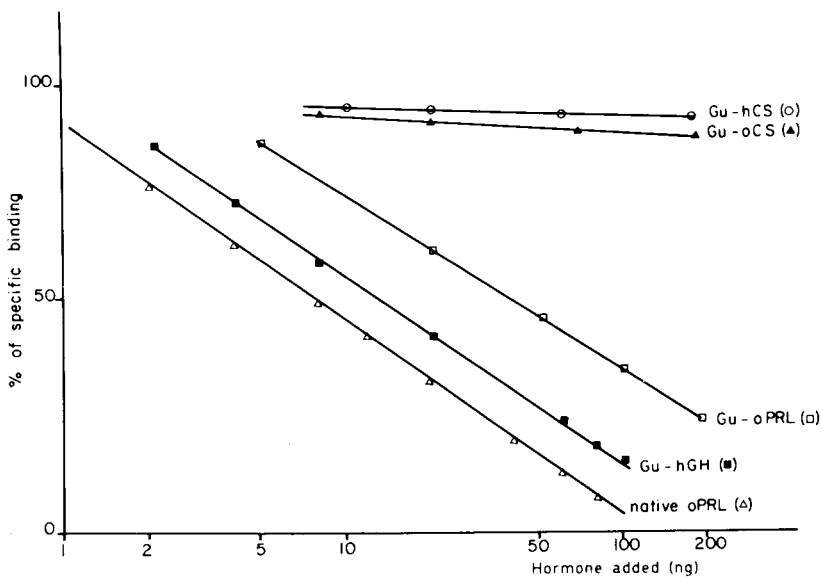


FIG. 4. — Specific binding of guanidinated hormones in a radioreceptor assay.

In figure 5, acetimidation led to a significant loss of activity for three hormones, only hGH molecule exhibited full activity.

Whatever the type and degree of modification, it may be assumed that lysine residues are involved in the binding capacity of these four hormones to lactogenic

receptors. A similar conclusion was obtained when studying somatotrophic activity of oCS (Chène *et al.*, 1984) and hGH (Martal *et al.*, 1985).

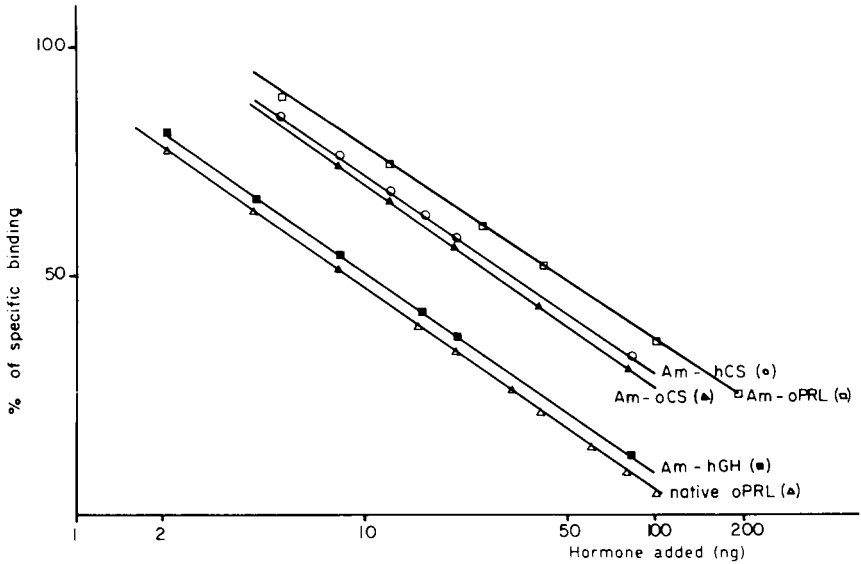


FIG. 5. — Specific binding of acetimidylated hormones in a radioreceptor assay.

Such modifications of the activity are depending on the protein structure, and the examination of aminoacid sequences of these different hormones in relation with lactogenic activity is envisaged in the present paper.

Primary structures of ovine (Li *et al.*, 1970), bovine (Wallis, 1974), porcine (Li, 1976), human (Shome and Parlow, 1977) and mouse PRL (Kohmoto *et al.*, 1984) were elucidated by protein sequencing. The primary sequences of rat (Cooke *et al.*, 1980) and human PRL (Cooke *et al.*, 1981) were recently investigated by complementary DNA sequencing.

Li (1978) established the primary structure of both hCS and hGH molecules. They found 85 % of identical aminoacids and 96 % homology as regards conservative mutations.

In table 2, we compared the aligned sequences of seven hormones : five prolactin hormones, hCS and hGH, according to the methods of Dayhoff (1972), and Martal (1980), taking gaps into account until aminoacids in position 62 ; afterwards disulfide bond between cysteins 62 and 180 imposes a rigid conformation to the molecule, N-terminal gaps being not involved. Only the position of lysine residues and sometimes arginine residues (a basic aminoacid) was considered. Some lysine residues were particularly well preserved during evolution : lysines 128 and 187, they are common to all prolactins (rat, pig, sheep, cattle, man), to hCS — the only placental lactogen whose primary structure was reported — and to all known growth hormones, such as human (Bewley *et al.*, 1972), ovine (Li *et al.*, 1972), pig (Mills and Wilhelmi, 1972), bovine (Wallis, 1973), rat (Wallis and Davies, 1976) and equine GH (Daurat-Larroque *et al.*, 1977).

TABLE 2
Compared primary sequences of different lactogenic hormones.

rPRL	Glu	Leu	Gly	Gly	Gly	Leu	Gly	Ala	Pro	Asp	Ala	Ile	Ile	Ser	Arg	Ala	Lys	Glu	Ile	Glu	Glu
bPRL	His	Leu	Val	Arg	Gly	Val	Arg	Ala	Pro	Asp	Ala	Ile	Leu	Ser	Arg	Ala	Ile	Glu	Glu	Glu	Glu
pPRL	His	Leu	Val	Arg	Gly	Val	Arg	Ala	Pro	Asp	Ala	Ile	Leu	Ser	Arg	Ala	Ile	Glu	Ile	Glu	Glu
oPRL	His	Leu	Val	Arg	Gly	Val	Arg	Ala	Pro	Asp	Ala	Ile	Leu	Ser	Arg	Ala	Ile	Glu	Ile	Glu	Glu
hPRL	His	Leu	Val	Arg	Gly	Val	Arg	Ala	Pro	Asp	Ala	Ile	Leu	Ser	Arg	Ala	Ile	Glu	Ile	Glu	Glu
hCS	Phe	Leu	Arg	Thr	Met	Phe	Ala	Thr	Tyr	Asp	Thr	Ser	Ser	Asp	Asp	Tyr	Tyr	His	Leu	*	*
hGH	Phe	Leu	Arg	Ser	Val	Phe	Ala	Val	Tyr	Gly	Ala	Ser	Asn	Ser	Asp	Val	Tyr	Asp	Leu	*	*
rPRL	Gln	Asn	Lys	Arg	Leu	Leu	Glu	Ile	Ile	Gly	Gln	Ala	Tyr	Pro	Glu	Ala	Lys	Gly	Asn	Glu	Ile
bPRL	Glu	Asn	Lys	Arg	Leu	Leu	Glu	Met	Phe	Gly	Gln	Val	Ile	Pro	Gly	Ala	Lys	Glu	Thr	Glu	Pro
pPRL	Glu	Asn	Lys	Arg	Leu	Leu	Glu	Ile	Val	Gly	Gln	Val	Ile	Pro	Gly	Ala	Lys	Glu	Glu	Glu	Val
oPRL	Glu	Asn	Lys	Arg	Leu	Leu	Glu	Met	Phe	Gly	Gln	Val	Ile	Pro	Gly	Ala	Lys	Glu	Thr	Glu	Pro
hPRL	Gln	Thr	Lys	Arg	Leu	Leu	Glu	Ile	Val	Ser	Gln	Val	Ile	Pro	Glu	Thr	Lys	Glu	Asp	Glu	Ile
hCS	*	Leu	Lys	Asp	Leu	Glu	Gly	Leu	Met	Gly	Arg	Leu	Glu	Asp	Gly	Ser	Arg	Arg	Thr	Gly	Gln
hGH	*	Leu	Lys	Asp	Leu	Glu	Gly	Leu	Met	Gly	Arg	Leu	Glu	Asp	Gly	Ser	Pro	Arg	Thr	Gly	Gln
rPRL	*	Tyr	Leu	Val	Trp	Ser	Gln	Leu	Gln	Gly	Val	Val	Asp	Glu	Ser	Lys	Asp	Leu	Ala	Phe	Tyr
bPRL	*	Tyr	Pro	Val	Trp	Ser	Gln	Leu	Gln	Thr	Lys	Lys	Asp	Glu	Asp	Arg	Tyr	Ser	Ala	Phe	Tyr
pPRL	*	Tyr	Ser	Val	Trp	Ser	Gln	Leu	Gln	Met	Ala	Ala	Asp	Glu	Asp	Arg	His	Ser	Ala	Phe	Tyr
oPRL	*	Tyr	Pro	Val	Trp	Ser	Gln	Leu	Gln	Thr	Lys	Lys	Asp	Glu	Asp	Arg	His	Ser	Ala	Phe	Tyr
hPRL	*	Tyr	Pro	Val	Trp	Ser	Gln	Leu	Gln	Met	Ala	Ala	Asp	Glu	Ser	Glu	Leu	Ser	Ala	Phe	Tyr
hCS	Ile	Leu	Lys	Gln	Thr	Ser	Lys	*	Asn	Ser	His	His	Asn	Glu	Leu	Leu	Leu	Asn	Asn	*	Tyr
hGH	Ile	Phe	Lys	Gln	Thr	Ser	Lys	*	Asn	Ser	His	His	Asn	Glu	Leu	Leu	Leu	Asn	Asn	*	Tyr
rPRL	Asn	Asn	Ile	Arg	Cys	Ser	His	Lys	Val	Asp	Asn	Tyr	Tyr	Leu	Phe	Leu	Arg	Cys	Glu	Ile	Val
bPRL	Asn	Leu	Leu	Arg	Asp	Ser	Ser	Lys	Ile	Asp	Asn	Tyr	Tyr	Leu	Lys	Leu	Asn	Cys	Arg	Ile	Val
pPRL	Asn	Leu	Leu	Arg	Asp	Ser	Ser	Lys	Ile	Asp	Asn	Tyr	Tyr	Leu	Lys	Leu	Asn	Cys	Arg	Ile	Val
oPRL	Asn	Leu	Leu	Arg	Asp	Ser	Ser	Lys	Ile	Asp	Asn	Tyr	Tyr	Leu	Lys	Leu	Asn	Cys	Arg	Ile	Val
hPRL	Asn	Leu	Leu	Arg	Asp	Ser	Ser	Lys	Ile	Asp	Asn	Tyr	Tyr	Leu	Lys	Leu	Asn	Cys	Arg	Ile	Val
hCS	Gly	Leu	Leu	Arg	Asp	Met	Asp	Lys	Val	Glu	Thr	Phe	Phe	Leu	Arg	Met	Val	Gln	Cys	Arg	*
hGH	Gly	Leu	Leu	Arg	Asp	Met	Asp	Lys	Val	Glu	Thr	Phe	Phe	Leu	Arg	Met	Val	Gln	Cys	Arg	*
rPRL	His	Lys	Asn	Asn	Cys	OH	OH	Lys	Val	Asp	Asn	Tyr	Tyr	Leu	Lys	Lys	Arg	Cys	Glu	Arg	Ser
bPRL	Tyr	Asn	Asn	Asn	Cys	OH	OH	Lys	Ile	Asp	Thr	Tyr	Tyr	Leu	Lys	Lys	Arg	Cys	Arg	Arg	Ser
pPRL	Tyr	Asn	Asn	Asn	Cys	OH	OH	Lys	Ile	Asp	Thr	Tyr	Tyr	Leu	Lys	Lys	Arg	Cys	Arg	Arg	Ser
oPRL	Tyr	Asn	Asn	Asn	Cys	OH	OH	Lys	Ile	Asp	Thr	Tyr	Tyr	Leu	Lys	Lys	Arg	Cys	Arg	Arg	Ser
hPRL	His	Asn	Asn	Asn	Cys	OH	OH	Lys	Ile	Asp	Thr	Tyr	Tyr	Leu	Lys	Lys	Arg	Cys	Arg	Arg	Ser
hCS	Val	Glu	Gly	Ser	Cys	OH	OH	Lys	Val	Glu	Thr	Phe	Phe	Leu	Arg	Met	Val	Gln	Cys	Arg	*
hGH	Val	Glu	Gly	Ser	Cys	OH	OH	Lys	Val	Glu	Thr	Phe	Phe	Leu	Arg	Met	Val	Gln	Cys	Arg	*

* Gap

Homologous Lysine or Arginine residues

Lysines in position 42 and 51 of PRL family could approximately correspond in hGH and hCS sequences to the positions of lysine 47 and 50 respectively, which could occupy a similar place in the steric conformation of the molecule when gaps are excluded. Nevertheless, this aminoacid residue 50 of hGH and hCS was preserved in all known growth hormones, but lysine 47 of hGH and hCS was substituted by a glutamic acid in ovine, bovine, rat and equine GH ; these latter hormones are not lactogenic.

Other prolactin lysines (73, 146, 193) are substituted by arginines in hGH (73, 147, 193) and in hCS (146, 193). As residue 73 exists in all the molecules exhibiting prolactin activity except in hCS, this basic residue could contribute to the activity without being indispensable.

Lysines 153 and 158 are common to hGH and hCS molecules, but they do not exist in PRL family, as arginines 170 and 183 of PRL family are substituted by lysines 172 and 183 in hCS and hGH hormones.

Birk and Li (1978) assumed that an enzymatic cleavage by plasmin of the oPRL peptide bond between Met 56 and Ala 57 destroys biological activity. Lactogenic activity was preserved after selective removal of residues 148-158 by trypsin (table 2), thus lysines 153 and 158 are not implicated in the lactogenic function (Graf *et al.*, 1982).

Graf and Li (1974) and Doneen (1975) showed that partial lactogenic activity was developed by fragment 1-147 of hGH (table 2) suggesting that lysines in position after 147 are not indispensable.

In *conclusion*, aminoacid residues of PRL family in positions 42, 51, 128, 146, in positions 47, 50, 128, 147 for hGH and for hCS might be essential for the binding to lactogenic receptors. It must be pointed out however that the presence of a basic residue at positions 50, 128, 147 in hGH is observed also in the case of other growth hormones suggesting that the basic residues at these positions are essential for binding but do not define the specificity of the molecule for lactogenic receptors.

Further studies will be required to locate and define precisely the contribution of these lysines and other aminoacids to the lactogenic activity.

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Résumé. *Hormones lactogènes : relations structure-fonction.*

L'activité lactogène de plusieurs dérivés hormonaux obtenus après modifications chimiques des résidus lysine a été analysée par dosage radio-hormone-récepteur. Les relations entre la structure des hormones modifiées et la liaison aux récepteurs lactogènes sont discutées en considérant les positions des résidus lysines susceptibles d'être impliqués dans la localisation de la fonction lactogène.

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