Influence of Mg^{2+} on detection of somatogenic and lactogenic components of growth-hormone-binding protein in mammalian sera

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We recently classified the growth-hormone (GH)-binding protein (GH-BP) in a wide range of mammalian [including human (h)] sera and reported the existence of a major lactogenic component in GH-BP of type-III sera (rabbit, horse, dog, pig and cat), based on the capacity of bovine (b) and ovine prolactin (PRL) to displace ^{125}I -labelled human growth hormone (hGH) binding and on direct ^{125}I -bPRL binding studies. In this study, we demonstrate the high degree of Mg^{2+} dependence of the binding of the classically lactogenic hGH and bPRL, but not that of the somatogenic bGH to various mammalian sera (types I–IV). Serum GH-BP was assayed using a previously described and validated charcoal-separation assay. ^{125}I -hGH binding to rat, ovine, bovine, rabbit, horse, dog and human sera was enhanced 1.5–2.5-fold in the presence of 70 mM Mg^{2+} . The Mg^{2+} effect was concentration-dependent between 3.7 mM and 70 mM, causing a significant and proportional increase in ^{125}I -hGH binding to serum. Like ^{125}I -hGH, ^{125}I -bPRL binding to type-III sera was also Mg^{2+} -dependent. In contrast, ^{125}I -bGH binding to all types

of serum GH-BP was not affected by Mg^{2+} concentrations of up to 35 mM, while 70 mM Mg^{2+} slightly, but significantly, reduced (by approx. 15%) bGH binding to rabbit serum. In keeping with the Mg^{2+} -dependent stimulation of lactogenic hormone binding to GH-BP, 70 mM Mg^{2+} caused a shift to the left in the displacement curves of hGH and bPRL competing with ^{125}I -hGH binding to rabbit, dog, horse and human sera, while the effects of the somatogens bGH and rabbit GH were shifted to the right. Scatchard analysis of hGH displacement curves with sera from various species yielded linear plots and revealed that Mg^{2+} significantly increased (2.3–3.0-fold) the affinity constants, but not the binding capacities. These results demonstrate the ability of changes in Mg^{2+} concentration to determine the degree of differential recognition of somatogens versus lactogens by serum GH-BP. It remains to be determined whether such bivalent cation effects may account, at least in part, for the growth retardation seen in Zn^{2+} or Mg^{2+} ion deficiencies.

INTRODUCTION

We recently reported the binding characteristics of human (h) growth hormone (GH) and bovine (b) prolactin (PRL) to sera of different mammalian species [1]. Sera were classified into four types, according to their binding affinity and specificity towards GHs and PRLs. An original finding of that study was the dominant lactogenic binding component contained in GH-binding protein (GH-BP) of sera from species defined as type-III sera (rabbit, horse, cat, dog and pig). One possible reason for the inability of previous studies to detect significant PRL binding to serum may revolve around the importance of cations for lactogenic binding, as previously reported [2–5]. In our preliminary binding studies we repeatedly observed that lower Mg^{2+} concentrations were associated with reduced lactogenic binding. Indeed, the cation dependence of hGH binding to rabbit liver membrane GH-receptor (GH-R) [6] and to GH-BP in rabbit serum [7] and cytosol [8] have been reported previously. In contrast, rat (r) GH, bGH and ovine (o) GH binding to rabbit liver membranes [2,5], cytosol or serum binding proteins [1] are cation independent. Furthermore, Zn^{2+} ions were shown to be required for hGH binding to PRL receptor (PRL-R) but not to GH-R, suggesting a fundamental dimorphism of these two receptors with regard to cation effects [9].

To more fully understand the cation dependence of GH and PRL binding to GH-BP in sera of various species, we have examined the effects of Mg^{2+} concentration on the binding properties and competitive potencies of hGH, bPRL and bGH.

MATERIALS AND METHODS

Hormones and other materials

Recombinant hGH and bGH were a gift from BioTechnology General Ltd. (Rehovot, Israel). bPRL (B-6) was obtained from the National Hormone and Pituitary Program, NIDDK, National Institutes of Health (Bethesda, MD, U.S.A.). Rabbit GH (AFP 10278B) was kindly obtained from Dr. A. F. Parlow (UCLA, Torrance, CA, U.S.A.). $Na^{125}I$ was purchased from the Nuclear Research Center–Negev (Beer-Sheva, Israel). Sephadex G-100 and dextran T-70 were purchased from Pharmacia (Uppsala, Sweden). BSA (fraction V) and Norit-A charcoal were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All other chemicals were of analytical grade and obtained from local commercial sources. Horse and bovine sera were commercial batches obtained from Biological Industries, Kibbutz Bet-Haemek, Israel.

In this study we also used sera obtained from adult Sprague–Dawley female rats, common European domestic male rabbits, dogs, sheep and adult human volunteers. Sera were immediately frozen upon separation and stored at $-20^{\circ}C$ until required for use.

Iodination

hGH, bPRL and bGH were radiolabelled using $Na^{125}I$ by the chloramine-T method [10] and chromatographed on a Sephadex G-100 column (45 cm $\times$ 1.5 cm). The column was eluted at $4^{\circ}C$

Abbreviations used: GH, growth hormone; PRL, prolactin; h, human; b, bovine; r, rat; o, ovine; rb, rabbit; GH-BP, GH binding protein; GH-R, GH receptor; PRL-R, PRL receptor; IGF-I, insulin-like growth factor I.

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Table 1 Effect of Mg^{2+} (70 mM) on ^{125}I -hGH binding to sera of various species

Values are expressed as means $\pm$ S.E.M. ($n = 4$). The serum dilution for each species was chosen to ensure that the specific binding would be within the linear portion of the volume-dependence curve.

Species	Serum dilution	^{125}I -hGH-specific binding (%/0.05 ml of diluted serum)	
		— Mg^{2+}	+ Mg^{2+}
Rat	1:1	3.8 ± 0.8	$9.5 \pm 0.9^*$
Sheep	1:1	3.7 ± 0.3	$9.1 \pm 1.0^*$
Cow	1:1	2.1 ± 0.3	$3.9 \pm 0.4^*$
Rabbit	1:4	14.2 ± 0.9	$25.5 \pm 2.0^*$
Dog	1:2	9.9 ± 0.5	$20.7 \pm 1.2^*$
Horse	1:2	16.3 ± 0.7	$26.0 \pm 1.5^*$
Human	1:1	6.3 ± 0.3	$11.9 \pm 0.5^*$

* $P < 0.05$ versus values in the absence of Mg^{2+} .

Figure 1. Mg^{2+} dependence of ^{125}I -hGH binding to rat (a), rabbit (b), dog (c) and human (d) serum

^{125}I -hGH (1 ng) was incubated with serum of female rat (0.05 ml), rabbit (0.0125 ml), dog (0.025 ml) and human (0.05 ml) in 0.01 M phosphate buffer with 1% (w/v) BSA, pH 7.4, and with increasing concentrations of Mg^{2+} ions. The serum dilution for each species was chosen to ensure that the specific binding would be within the linear portion of the volume-dependence curve. Values are expressed as percentage of specific binding/0.05 ml of serum and represent means $\pm$ S.E.M. of three separate experiments, each performed in duplicate.

with 0.01 M phosphate buffer, containing 1% (w/v) BSA, pH 7.4. The specific radioactivity ranged from 60–80 Ci/g.

Assay methods

Binding studies were usually carried out at 4 °C for 24 h using 0.01 M phosphate buffer containing 1% (w/v) BSA, pH 7.4, in

Figure 2. Mg^{2+} dependence of ^{125}I -bPRL (a) and ^{125}I -bGH (b) binding to rabbit serum

Rabbit serum (0.05 ml) was incubated with ^{125}I -bPRL (1 ng) or ^{125}I -bGH (1 ng) in the absence or presence of excess unlabelled bGH or bPRL respectively and increasing concentrations of Mg^{2+} ions. Values are expressed as percentage of specific binding/0.05 ml of serum and represent the means $\pm$ S.E.M. of three separate experiments, each performed in duplicate.

the absence or presence of increasing concentrations (up to 70 mM) of added $MgCl_2$ in a final volume of 270 μ l, essentially as described previously [11,12]. ^{125}I -hGH, ^{125}I -bPRL or ^{125}I -bGH (1 ng) were incubated with 50 μ l of serum at the appropriate dilution, in the absence (total binding) or presence (non-specific binding) of 1 μ g of their respective unlabelled hormones. For each binding assay and species, the dilution of the serum was chosen to ensure that specific binding was within the linear part of the volume-dependence binding curve (results not shown). After incubation, bound and free hormones were separated by adding 1 ml of dextran-coated charcoal {2% (w/v) Norit A, 0.2% dextran T-70 in 0.01 M phosphate buffer, pH 7.4, for ^{125}I -hGH binding studies, or 3% (w/v) Norit A, 0.3% dextran T-70 in 0.1 M phosphate buffer, pH 7.4, for ^{125}I -bPRL and ^{125}I -bGH binding studies [1,11,12]}.

Calculations

Binding affinity constants and capacities were estimated by Scatchard analysis [13] of binding data derived from dose-response studies using increasing concentrations of unlabelled hormone with a fixed concentration of labelled hormone. The potency of each hormone was estimated by determining the IC_{50} ,

which defines the concentration of the respective unlabelled hormone preparation that reduced to 50% the ¹²⁵I-hGH specifically bound to sera. Statistical analysis of results was based on the paired two-tailed Students *t*-test, comparing experimental group means $\pm$ S.E.M. for testing of significance. The significance level was set at $P < 0.05$.

RESULTS

Effect of Mg²⁺ on ¹²⁵I-hGH, ¹²⁵I-bPRL and ¹²⁵I-bGH binding to sera of various species

¹²⁵I-hGH-specific binding to rat, ovine, bovine, rabbit, dog, horse and human sera was enhanced 1.6–2.5-fold in the presence of

Table 2 IC₅₀ values of various hormones in the displacement of ¹²⁵I-hGH binding from sera of various species in the absence or presence of Mg²⁺ (70 mM)

Values are expressed as ng/tube and are the means $\pm$ S.E.M. from three separate experiments, each performed in duplicate. * $P < 0.05$ versus value in the absence of Mg²⁺. Abbreviation: N.C., non-competing for ¹²⁵I-hGH binding.

Species	Hormone	IC ₅₀ value	
		– Mg ²⁺	+ Mg ²⁺ (70 mM)
Rabbit	hGH	2.2 $\pm$ 0.2	1.4 $\pm$ 0.3
	bPRL	18.0 $\pm$ 1.9	4.1 $\pm$ 0.9*
	bGH	3.6 $\pm$ 1.4	13.0 $\pm$ 2.1*
	rbGH	440.0 $\pm$ 28.0	> 1000
Dog	hGH	2.1 $\pm$ 0.3	1.2 $\pm$ 0.3
	bPRL	17.2 $\pm$ 1.9	8.1 $\pm$ 1.2*
	bGH	3.5 $\pm$ 0.4	6.0 $\pm$ 0.8*
Horse	hGH	4.2 $\pm$ 0.6	1.6 $\pm$ 0.3*
	bPRL	27.1 $\pm$ 1.0	11.2 $\pm$ 1.6*
	bGH	3.7 $\pm$ 0.6	8.5 $\pm$ 1.4*
Human	hGH	22.0 $\pm$ 1.5	10.1 $\pm$ 1.1*
	bPRL	> 1000	240.0 $\pm$ 17.0
	bGH	N.C.	N.C.

Figure 3. Hormonal specificity of binding of ¹²⁵I-hGH to rabbit serum in the presence (a) and absence (b) of 70 mM Mg²⁺

¹²⁵I-hGH (1 ng) was incubated with rabbit serum (0.0125 ml) in the presence (a) or absence (b) of Mg²⁺ (70 mM) and in the absence or presence of increasing concentrations of unlabelled competing hormones. Maximal absolute values of ¹²⁵I-hGH-specific binding in the presence or absence of Mg²⁺ were 18.5%/0.05 ml of serum and 8.9%/0.05 ml serum respectively and these values served as the 100% controls. Values are the means of triplicate determinations from a representative experiment that was repeated three times.

added Mg²⁺ ions (Table 1). In the absence of added Mg²⁺, the contribution of Mg²⁺ by the various sera ranged between 70 and 180 μ M, based on their maximal and minimal dilution in the assay incubation mix and on the reported physiological Mg²⁺ levels for the various serum species [14].

The dose-response curves for the effects of Mg²⁺ concentration on the binding of ¹²⁵I-hGH to rat, rabbit, dog and human sera are shown in Figure 1. Mg²⁺ concentrations of 3.7–70 mM significantly stimulated ¹²⁵I-hGH-specific binding to sera from various species. Like ¹²⁵I-hGH, ¹²⁵I-bPRL-specific binding to serum was also shown to be Mg²⁺-dependent (Figure 2a). In contrast with hGH and bPRL binding, ¹²⁵I-bGH binding to serum GH-BP was not affected by Mg²⁺ concentrations of up to 35 mM, while 70 mM Mg²⁺ even significantly reduced the binding to bGH to rabbit serum (Figure 2b).

Hormonal specificity of binding of ¹²⁵I-hGH to serum GH-BP: Influence of Mg²⁺

Figure 3 shows the competition of hGH, bGH, rbGH and bPRL for ¹²⁵I-hGH binding to rabbit serum in the absence or presence of added Mg²⁺ ions. Mg²⁺ induced a shift to the right in the bGH and rbGH displacement curves, while those of hGH and bPRL were shifted to the left; bPRL was significantly more potent, while bGH was less potent, when the incubation reaction mixture contained Mg²⁺ than in the absence of added Mg²⁺ (Table 2). The homologous ligand rbGH was also more potent at competing with ¹²⁵I-hGH in the absence of added Mg²⁺. A similar pattern of Mg²⁺-dependent shift in hormonal competition was also observed in dog and horse sera (Table 2). Moreover, in human serum, as in rabbit serum, bPRL was approx. 4-fold more potent in the presence of added Mg²⁺ (Table 2).

Effect of Mg²⁺ on hGH-binding capacity and affinity to serum GH-BP

A representative Scatchard analysis of hGH binding to rabbit serum in the absence or presence of added Mg²⁺ (70 mM) is shown in Figure 4. Scatchard analysis [13] of the results, obtained

Figure 4. Scatchard plots of ^{125}I -hGH binding to rabbit serum in the presence and absence of 70 mM Mg^{2+}

Representative Scatchard analysis of ^{125}I -hGH binding to rabbit serum in the presence (●) and absence (○) of Mg^{2+} (70 mM). The ordinate represents the ratio of bound/free hGH (B/F) and the abscissa the number of fmol bound/ml of serum. The points are the means of triplicates from an experiment that was repeated three times with similar results (see Table 3).

Table 3 Scatchard analysis of ^{125}I -hGH binding to sera of various species in the absence or presence of Mg^{2+} (70 mM)

Values are the mean $\pm$ S.E.M. of three separate experiments each performed in duplicate. * $P < 0.05$ versus value in absence of Mg^{2+} .

Species	– Mg^{2+}		+ Mg^{2+}	
	$10^9 \times K_a$ (M^{-1})	N (fmol/ml)	$10^9 \times K_a$ (M^{-1})	N (fmol/ml)
Rabbit	2.95 ± 0.38	4027 ± 113	$8.95 \pm 0.29^*$	3694 ± 106
Dog	5.50 ± 0.52	374 ± 24	$14.22 \pm 1.25^*$	491 ± 39
Horse	4.28 ± 0.61	882 ± 72	$11.10 \pm 1.23^*$	908 ± 24
Human	0.45 ± 0.09	872 ± 31	$1.02 \pm 0.18^*$	882 ± 74

from displacement curves of hGH binding to sera of various species, yielded linear plots. The affinity constants (K_a) in the presence of Mg^{2+} were significantly higher (2.3–3.0-fold), while binding capacity was not significantly changed (Table 3).

DISCUSSION

The present study demonstrates the very high degree of Mg^{2+} dependence of hGH and bPRL binding to various mammalian sera in the face of a lack of such ionic dependence for bGH binding. Recently, the existence of a lactogenic component in GH-BP of sera from rabbit, horse, dog, pig and cat was demonstrated, based on the capacity of bPRL and oPRL to displace hGH binding, and on direct ^{125}I -bPRL-specific binding studies [1]. In fact, Mg^{2+} potentiated hGH binding to GH-BP in

each of the four groups of species recently classified according to hGH- and bPRL-binding levels, binding specificity towards GHs and PRLs, binding affinity and the ability of a monoclonal anti-(rabbit bGH-R) antibody to inhibit the binding to serum [1]. For example, Mg^{2+} dependence of hGH binding was observed in the rat, which had serum GH-BP classified as type-I (low affinity for hGH and 'purely' somatogenic binding, based on lack of effects of PRLs on hGH binding [1,12]). It was also observed in the rabbit, horse and dog, which were included among type-III sera (high affinity for hGH and a strong lactogenic component), and it was found in human (type-IV serum), which is characterized by the inability of non-primate GHs to efficiently compete with hGH binding [11,15]. These findings of cation dependence of hGH binding to GH-BP in various mammalian sera are in accordance with previously described effects of Ca^{2+} or Mg^{2+} on hGH binding to rabbit liver membrane homogenates, detergent- or aqueous-solubilized membranes, cytosolic BPs and rabbit serum [2,4–8]. More recently, D'Abronzio et al. [16] reported dose-dependent effects of Ca^{2+} and Mg^{2+} on ^{125}I -hGH binding to human liver microsomes. Furthermore, our parallel observation of Mg^{2+} independent of bGH binding to the various sera tested here is in keeping with the similar cation independence described in rabbit liver membranes [5], cytosolic BPs and rabbit serum [2]. In the current studies, analysis of the Scatchard plots of hGH-binding studies showed that Mg^{2+} had no effect on the number of serum binding sites, but it did result in higher binding affinity. This is in keeping with the increase observed, in the presence of 25 mM Ca^{2+} , in hGH-binding affinity to female rabbit liver cytosol [4].

The similarity in cation dependence of hGH binding to GH-BP with that to membrane GH-R and cytosolic BPs is consistent with the sequence identity shown to exist between GH-BP and the extracellular domain of the hepatic GH-R [17], and further supports the proposition that measurement of serum GH-BP may reflect upon membrane GH-R function. Nevertheless, the divergent cation-dependent curve shifts observed in the competitive-binding studies further stress the difference between those hormones classically identified as lactogenic and those identified as somatogenic. Thus, when the incubation reaction contained Mg^{2+} hGH and bPRL were significantly more potent, whereas bGH and rbGH were less potent, at inhibiting ^{125}I -hGH binding to rabbit serum, compared with their effects in the absence of added Mg^{2+} , and vice versa. These results could be explained by postulating the existence of a single type of serum binding site recognizing lactogenic hormones (hGH, PRLs) preferentially in the presence of added Mg^{2+} and somatogenic hormones (all GHs) in its absence. Such a concept was originally proposed to explain findings which suggested the existence of a 'shared' GH-R for both lactogenic and somatogenic hormones in rabbit liver membranes [18,19], and invoked more recently by Ciccia-Torres and Dellacha [3] who also raised the possibility of 'shared' binding sites existing in the mouse liver, that bound lactogenic and somatogenic ligands with different cation dependencies. Thus, it is conceivable that in parallel with the 'shared' hepatic GH-R, the serum GH-BP might also be a 'shared' GH-BP, which binds both GH and PRL. However, it is noteworthy that the presence/absence of added Mg^{2+} in the incubation reaction could not cause a non-competing hormone to become a competing hormone for hGH binding. For example, bGH does not become a competing hormone for hGH binding to human serum, even in the absence of added Mg^{2+} , and bPRL does not inhibit hGH binding to rat serum, even in the presence of a high Mg^{2+} concentration. Similarly, even in the presence of Mg^{2+} no specific binding of ^{125}I -bPRL to rat serum was observed (results not shown). Thus, in subsequent studies *in vitro* on serum GH-

BPs it would be important to carefully monitor the cation composition of the incubation mixture, since bivalent cations may favour, to various extents, the binding of hormones with lactogenic activity, while their absence allows the recognition of binding sites with somatogenic specificity.

Barnard and Waters have proposed an electrostatic model to illustrate the cation dependence, in terms of a total involvement of cations in the formation of the hormone BP complex [4]. That model invoked convergence of unit negative charges on the hormone and binding protein towards an intercalated Ca²⁺ ion, which could also account for the observed cation-mediated increase in hGH-binding affinity. A positive charge shown to exist in the critical region of non-primate GHs, would render their interaction with the binding protein Ca²⁺-independent. Nevertheless, these findings do not rule out the suggestion that the divergence in cationic effect resides with the different hormones, rather than with their receptors. This suggestion, however, is not intended to reduce the importance of the finding of fundamentally different metal-ion requirements for hGH binding to GH-R and to PRL-R, since Zn²⁺ was shown to be required for tight binding of hGH to the hPRL-R, but not for binding to the GH-R [9].

When considering the physiological implications of the *in vitro* studies reported here, it should be stressed that the Mg²⁺ concentrations used here (3.7–70 mM, covering the range normally employed in similar binding studies *in vitro*) are in the high to supraphysiological range for the mammalian sera studied (range: 1.3–5.4 mg/dl equivalent to 0.5–2.3 mM for rabbit, dog, horse, rat and human [14]). Thus, only the lowest (but nevertheless effective) concentration tested approaches the highest physiological serum levels reported and therefore the major implication of our findings will be for studies of GH/PRL binding *in vitro*. Nevertheless, it is possible that, *in vivo*, even lower Mg²⁺ concentrations, by acting in concert with other cations such as Zn²⁺ and Ca²⁺, might exert physiologically relevant regulatory effects on GH/PRL binding to serum GH-BP and to GH/PRL receptors. In a rat model, Zn²⁺ and Mg²⁺ deficiencies were recently reported to be accompanied by reduced circulating insulin-like growth factor I (IGF-I) (which can be seen as reflecting the response to GH) in the face of unchanged serum GH levels [20]. Since, in our hands, Mg²⁺ did not appear to affect the non-primate, purely somatogenic GHs at the level of the hormone, it must be postulated that, in the rat, the cation deficiency reduced the GH effects of IGF-I by acting on the level of, or distally to, the GH-R. However, in the human, it remains

to be determined whether cationic effects on hGH binding to GH-BP/GH-R, in addition to a possible regulatory effect at the level of the receptor or distal to it, may be involved in the growth retardation seen in Zn²⁺ or Mg²⁺ deficiencies, in view of the finding that, in GH-deficient children, Zn²⁺ supplementation improved the growth response to administered GH [21].

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