Comparison of Lipolytic and Antilipolytic Activities of Lower Vertebrate Growth Hormones on Chicken Adipose Tissue *In Vitro* (43275)

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Abstract. **Mammalian and avian growth hormones (GH) (pituitary derived or biosynthetic) exert two effects on chicken adipose tissue explants *in vitro*. They (i) increase the basal rate of glycerol release a lipolytic effect) and (ii) inhibit glucagon-stimulated glycerol release (an antilipolytic effect). The ability of lower vertebrate GH preparations to exert lipolytic and antilipolytic effects was examined and biological activity was compared to differences in amino-acid residue sequences and to predicted structure. Irrespective of species origin (blue shark, sturgeon, bonito, yellow tail, salmon, bullfrog, sea turtle), all lower vertebrate GH preparations showed very weak (<5% the potency of bovine GH), if any, lipolytic activity, but retained strong antilipolytic activity. The present data indicate that the structural requirements for lipolytic and antilipolytic activities of GH differ in chicken adipose tissue. Despite the high sequence homology (88%) between chicken and sea turtle GH, the latter preparation did not stimulate lipolysis. It is suggested that Pro^{132}, conserved only in lipolytically active GH species (human, bovine, and chicken), represents a major determinant of lipolytic activity in chicken adipose tissue. The structural determinants for antilipolytic activity may comprise any or all of residues 3, 17, 64, 108, 109, and 152.** [P.S.E.B.M. 1991, Vol 197]

Growth hormone (GH) has been purified from many disparate nonmammalian vertebrate species (e.g., blue shark [1], sturgeon [2], chum salmon [3], yellow tail [4], bonito [5], tuna [6], bullfrog [7], sea turtle [7], and chicken [8, 9]). For the vast majority of these growth hormones, the sequence of amino acid residues has been determined either directly from the purified protein (blue shark [10], bonito [6], bullfrog [11], sea turtle [12]) or from the cDNA (chum salmon [13], yellow tail [14], tuna [15], bullfrog [16], and chicken [17]). The availability of various GH with known amino acid sequences allows for examination of the relationship between GH structure and lipolytic and/or antilipolytic effects on chicken adipose tissue explants *in vitro*.

We have demonstrated previously that chicken, bovine, human 22-kDa GH, and human 20-kDa GH increase glycerol release (a lipolytic effect) and inhibit glucagon-induced glycerol release (an antilipolytic effect) from chicken adipose tissue *in vitro* (18–20). Bovine and human GH are 77% and 58% identical in sequence with chicken GH, respectively. However, human placental lactogen, which is 54% homologous with chicken GH, displays neither lipolytic nor antilipolytic activity in this assay (20). To ascertain the structural determinants of GH necessary for lipolytic and antilipolytic effects, a limited sequence database consisting of naturally occurring GH homologs has been constructed (i.e., correlating conserved amino acid residues with activity). In the present studies, the lipolytic and antilipolytic effects of blue shark, sturgeon, chum salmon, yellow tail, bonito, tuna, bullfrog, and sea turtle GH are examined. These GH preparations possess 64%,

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sturgeon (sequence not known), 39%, 38%, 38%, 37%, 64%, and 88%, respectively, sequence identity with chicken GH (using Fig. 1 sequence alignments).

Materials and Methods

In all studies, abdominal adipose tissue from adult (24- to 30-week-old) male chickens (Single Comb White Leghorn strain) was employed. Prior to sacrifice, birds were housed in brooder pens with free access to water and feed (Agway Chick Grower diet). Explants were prepared and incubated as described previously [18, 19). Briefly, this entailed dicing adipose tissue (from three chickens per trial) and distributing it among incubation vials (7–10 explants/vial, 40–80 mg total weight). Adipose tissue explants were incubated in 1 ml of Krebs-Ringer-HEPES medium (pH 7.4) supplemented with 15 m*M* glucose, 1% bovine serum albumin (Fraction V; Armour), and 2.54 m*M* calcium chloride under 95% O_2–5% CO_2 in a shaking water bath (70 oscillations/min, 37.5°C). Following a 1-hr preincubation, the medium was removed and fresh medium, containing test hormones, was added. Tissues were incubated for 1 hr and removed from the vials (for weight determination), and the medium was rapidly frozen. Medium glycerol content was determined by enzymatic/fluorometric assay (18) and used as the index of lipolysis. In all studies, biosynthetic bovine GH, whose lipolytic and antilipolytic activities have been defined in this *in vitro* model (18, 19), was included for comparison.

The purification of the GH preparations are described in detail elsewhere: blue shark (1), sturgeon (2), chum salmon (3), yellow tail (4), bonito (5), tuna (6), bullfrog (7), and sea turtle (7). Glucagon was obtained from Sigma Chemical Co. (St. Louis, MO).

Statistical differences between means were determined by analysis of variance, followed by Fisher's protected least significant differences. Biological potencies for GH preparations in Tables I through IV were determined by a Parallel Line Bioassay Computer Program (PARATL; Hoffmann-La Roche) using data from all doses, with bovine GH (potency, 1.00) as the standard for comparison. In all cases, significant ($P < 0.05$) parallelism was observed with the bovine GH standard. For yellow tail GH, in which case only one dose was tested because of supply limitations (Tables II and IV),

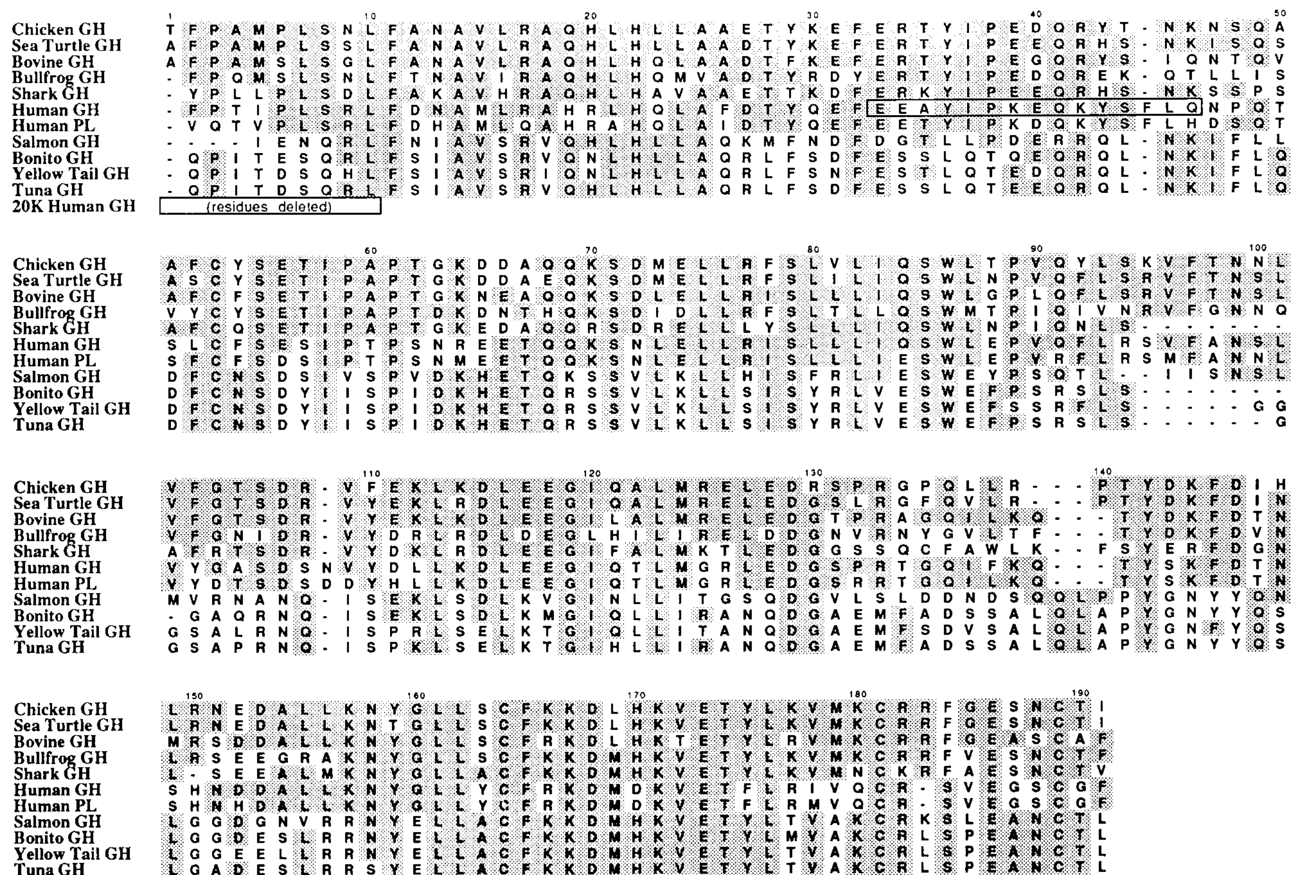

Figure 1. Comparison of amino acid sequences examined for lipolytic and/or antilipolytic activity in chicken adipose tissue: chicken (17), sea turtle (12), bovine (44), bullfrog (11, 16), blue shark (10), human (45, 46), 20-kDa human variant (47), salmon (13), bonito (6), yellow tail (14), and tuna (15) GH and human placental lactogen (human PL) (46). The full sequence of sturgeon GH has yet to be determined. Sequences were aligned using theoretical deletions to maximize homology. Amino acid residues are numbered according to the chicken GH sequence. Identical residues occurring in multiple (≥3) species are shaded.

Table I. Effect of Sturgeon, Bullfrog, Shark, Sea Turtle, Tuna, and Bovine GH on Chicken Adipose Tissue Lipolysis *In Vitro*

		Glycerol release during 1-hr incubation (nmol/g tissue ± SE, $n = 3$)[a]		
		0.01 μg/ml	0.1 μg/ml	1 μg/ml
Control	207.8 ± 27.9			
Bovine GH		282.0 ± 10.0[b]	331.0 ± 20.0[c]	382.6 ± 31.9[c]
Sturgeon GH		210.3 ± 24.1	173.8 ± 17.8	173.2 ± 16.7
Bullfrog GH		216.3 ± 18.4	174.5 ± 20.6	160.4 ± 21.4
Blue shark GH		215.6 ± 19.5	203.0 ± 17.8	164.2 ± 14.6
Sea turtle GH		218.0 ± 15.3	213.0 ± 12.6	217.6 ± 12.5
Tuna GH		227.7 ± 14.9	171.6 ± 14.1	139.9 ± 19.3[b]

[a] Values represent mean ± SE of three independent trials, with five replicates/treatment group/trial. Statistical differences were determined by analysis of variance, followed by Fisher's protected least significant differences.
[b] $P < 0.01$, compared with control.
[c] $P < 0.001$, compared with control.

Table II. Effect of Salmon, Bonito, Yellow Tail, and Bovine GH on Chicken Adipose Tissue Lipolysis *In Vitro*

		Glycerol release during 1-hr incubation (nmol/g tissue ± SE, $n = 3$)[a]		
		0.1 μg/ml	0.5 μg/ml	1 μg/ml
Control	243.0 ± 17.1			
Bovine GH		383.1 ± 10.3[b]	nt[c]	456.8 ± 9.1[b]
Salmon GH		269.4 ± 8.3	nt	342.7 ± 7.1[b]
Bonito GH		278.5 ± 12.7	nt	352.4 ± 26.8[b]
Yellow tail GH		nt	286.7 ± 11.6[d]	nt

[a] Values represent mean ± SE of three independent trials, with six replicates/treatment group/trial. Statistical differences were determined by analysis of variance, followed by Fisher's protected least significant differences.
[b] $P < 0.001$, compared with control.
[c] nt, not tested due to supply limitations.
[d] $P < 0.05$, compared with control.

Table III. Effect of Sturgeon, Bullfrog, Shark, Sea Turtle, Tuna, and Bovine GH on Glucagon-Induced Lipolysis by Chicken Adipose Tissue *In Vitro*

		Glycerol release during 1-hr incubation (nmol/g tissue ± SE, $n = 3$)[a]		
			Glucagon (1 ng/ml)	
		0.01 μg/ml	0.1 μg/ml	1 μg/ml
Control	183.4 ± 6.8			
Glucagon (1 ng/ml)	697.9 ± 15.0			
Bovine GH		582.4 ± 24.0[b]	496.3 ± 28.5[b]	443.1 ± 18.8[b]
Sturgeon GH		580.0 ± 13.6[b]	494.5 ± 12.1[b]	444.4 ± 8.1[b]
Bullfrog GH		626.2 ± 9.7[c]	560.3 ± 11.5[b]	491.2 ± 14.1[b]
Blue shark GH		587.9 ± 13.0[b]	540.9 ± 12.4[b]	456.7 ± 23.2[b]
Sea turtle GH		606.5 ± 2.0[c]	536.9 ± 11.2[b]	468.4 ± 3.3[b]
Tuna GH		611.4 ± 11.8[c]	544.3 ± 23.1[d]	479.3 ± 12.6[b]

[a] Values represent mean ± SE of three independent trials, with five replicates/treatment group/trial. All GH preparations were added at the concentrations above (0.01–1 μg/ml) in the presence of glucagon (1 ng/ml). Statistical differences were determined by analysis of variance, followed by Fisher's protected least significant differences.
[b] $P < 0.001$, compared with glucagon treatment alone.
[c] $P < 0.05$, compared with glucagon treatment alone.
[d] $P < 0.01$, compared with glucagon treatment alone.

potencies could not be calculated with PARATL (minimum $n > 1$). As the dose-response curves for all other GH (bovine, salmon, and bonito) were parallel (*i.e.*, displaying the same slope), an extrapolated dose-response curve for yellow tail GH was plotted using the average parallel slope to crudely estimate potency.

Results

Tables I and II summarize data on the ability of lower vertebrate GH preparations to stimulate glycerol release from chicken adipose tissue *in vitro*. As has been observed previously (18), bovine GH (0.01, 0.1, and 1 μg/ml) stimulated glycerol release from chicken adipose

Table IV. Effect of Salmon, Bonito, Yellow Tail, and Bovine GH on Glucagon-Induced Lipolysis by Chicken Adipose Tissue *In Vitro*

	Glycerol release during 1-hr incubation (nmol/g tissue ± SE, $n = 3$)[a]			
		Glucagon (1 ng/ml)		
		0.1 μg/ml	0.5 μg/ml	1.0 μg/ml
Control	271.9 ± 15.8			
Glucagon (1 ng/ml)	903.8 ± 22.1			
Bovine GH		638.6 ± 15.8[b]	nt[c]	571.9 ± 13.1[b]
Salmon GH		590.6 ± 11.4[b]	nt	527.5 ± 10.1[b]
Bonito GH		588.4 ± 13.7[b]	nt	522.5 ± 10.2[b]
Yellow tail GH		nt	523.7 ± 10.7[b]	nt

[a] Values represent mean ± SE of three independent trials, with five replicates/treatment group/trial. All GH preparations were added at the concentrations above (0.1, 0.5, and/or 1 μg/ml) in the presence of glucagon (1 ng/ml). Statistical differences were determined by analysis of variance, followed by Fisher's protected least significant differences.
[b] $P < 0.001$, compared with glucagon treatment alone.
[c] nt, not tested due to supply limitations.

tissue explants in a dose-dependent manner. However, preparations of GH from sea turtle, bullfrog, sturgeon, and blue shark failed to increase lipolysis. Salmon, bonito, and yellow tail GH were weakly lipolytic, with potencies estimated at 3.0%, 3.7%, and <1% that of bovine GH, respectively. In the case of tuna GH, decreased lipolysis (33%; significant at 1 μg/ml only) was observed.

In contrast to their very low or nonexistent lipolytic activities, the lower vertebrate GH preparations all displayed the ability to inhibit (≤36%) glucagon-induced glycerol release (Tables III and IV). Within 95% confidence limits (CL) (potency [95% CL: lower limit → upper limit]), sturgeon (1.03 [95% CL: 0.43 → 2.33]), blue shark (0.78 [95% CL: 0.31 → 1.22]), sea turtle (0.67 [95% CL: 0.30 → 1.19]), and tuna (0.58 [95% CL: 0.20 → 1.33]) GH were similarly potent in antilipolytic activity compared with the bovine GH standard (potency, 1.00). Bullfrog GH was significantly less potent (0.22 [95% CL: 0.04 → 0.49]) than bovine GH. Because the bullfrog GH displayed some aggregation in purification (Dr. J. Kostyo, personal communication), it is expected that the potency of bullfrog GH is actually higher than that observed. Salmon (6.09 [95% CL: 2.80 → 15.34]), bonito (6.09 [95% CL: 2.86 → 18.88]), and yellow tail (14.80 [estimated]) GH appeared to be more potent in the antilipolytic assay than bovine GH.

Discussion

In the present studies, poikilotherm GH preparations exhibited strong antilipolytic activity, but only weak, if any, lipolytic activities. These results may reflect a divergent evolution of the structural determinants (of GH) necessary for induction of lipolytic and antilipolytic responses. The data also suggest that the chicken adipocyte GH receptor responsible for the lipolytic activity of GH has different structural requirements than that for the antilipolytic activity. While this would be unexpected from the perspective of a single GH receptor, recent characterization/purification studies have lent credibility to the existence of multiple receptors and/or receptor subunits (liver [21, 22]—mouse [23], rabbit [24]; and adipose tissue—rabbit [24], rat [25]. In addition, GH fragments or variants that retain GH activity in some systems, but are not active or have very low activity in other systems, have been reported previously. For example, a reduced, carbamidomethylated human GH fragment (noncovalent complex of residues 1–134 and 141–191) is equipotent with human GH in the *in vivo* rat tibia test (26), *in vitro* lipolytic assay (27), and *in vitro* antilipolytic assay (27), but is only ≈16% as active as human GH in stimulating ^{14}C-glucose oxidation *in vitro* by adipose tissue from hypophysectomized rats (27). The 20-kDa variant of human GH (deletion of residues 32–46) has full rat tibia (28) lipolytic and antilipolytic activity (20), but very low insulin-like activity (29, 30).

The disparate antilipolytic and lipolytic activities of lower vertebrate GH suggest the possibility of two receptor subtypes. It may be questioned whether separate postreceptor signal transduction mechanisms exist. There is pharmacological evidence in chicken adipose tissue *in vitro* to support this concept. In chicken adipose tissue, the lipolytic, but not antilipolytic, effects of GH are blocked in the presence of protein synthesis inhibitors (cycloheximide, puromycin) (31), an RNA synthesis inhibitor (actinomycin D) (31), a calcium antagonist (verapamil) (31), cyclic GMP (8-bromo-cGMP, nitroprusside) (32), or incubation with calcium-free (31) or low-glucose (15 or 150 μ*M*) medium (33). Conversely, if polyamine synthesis is inhibited by α-difluoromethylornithine, then the antilipolytic effects of GH are not observed while the lipolytic effects are

still manifest (31). Studies with rat adipose tissue *in vitro* provide additional support for more than one receptor-effector system. Similar to chicken adipose tissue, inhibitors of RNA and protein synthesis block the lipolytic (i.e., GH + dexamethasone) (34), and not the antilipolytic, effect (i.e., inhibition of epinephrine-stimulated lipolysis) of GH in rat adipose tissue *in vitro* (35).

The relative inactivity of lower vertebrate GH preparations in the avian (chicken) adipose tissue lipolytic assay contrasts with those reported using mammalian bioassays. For example, many of these GH exhibit significant activity in the rat tibia test (sturgeon [3], blue shark [36], sea turtle [7], and bullfrog [7, 37]). While teleost GH is generally thought to be inactive in the rat tibia assay [38], GH bioactivity is observable in some cases, albeit at high doses (e.g., in the rat bioassay with tilapia GH [39] or chum salmon [40]). There is a single report (41) on the biological activities of lower vertebrate GH in two assays: (i) *in vivo* diabetogenic activity (fasting hyperglycemia, decreased glucose tolerance) in obese *ob/ob* mice, and (ii) *in vitro* insulin-like effects (increased ^{14}C-glucose oxidation by adipose tissue from hypophysectomized rats). Sea turtle, bullfrog, and sturgeon GH were active in both systems, although doses 20 to 1000-fold that of mammalian GH were required to evoke a response (41). Comparatively, there is much similarity between the lipolytic activity with chicken adipose tissue and the insulin-like activity of some of the lower vertebrate GH in rat adipose tissue *in vitro.* In both of these bioassays, sea turtle, bullfrog, and sturgeon GH have low potencies (≤5%) compared with that of mammalian GH.

It should be noted that, following the recent sequencing of sea turtle GH, the Met^5-residue was found to be oxidized (12). It was later determined that the Met^5 oxidation occurred as a result of storage conditions (H. Kawauchi *et al.*, unpublished observations). It might be argued that the lack of lipolytic activity with sea turtle GH reflects only the properties of the oxidized, and not that of the intact, protein. The current evidence appears to discount this idea, as sea turtle GH exhibited substantial antilipolytic activity in chicken adipose tissue *in vitro* (Table III). Moreover, all of the other lower vertebrate GH preparations examined were active in the antilipolytic assay, but were relatively inactive in the lipolytic assay. The cumulative data, with or without sea turtle GH, indicates that amino acid residue 5 is not a major determinant for lipolytic activity. The Met^5 residue is not conserved in lipolytic species such as human GH (Ile substitution), and is conserved in nonlipolytic species such as the shark (Table I).

The chicken adipose tissue GH receptor/receptor subunit responsible for the lipolytic effects of GH is highly sequence selective. The selectivity of the chicken adipose GH receptor/receptor subunit is exemplified by its ability to differentiate between lipolytic and nonlipolytic electrophoretic variants/isomers (equivalent molecule weight) of chicken GH (42, 43). It follows that small changes in GH sequence can result in the diminution of lipolytic activity. Sea turtle GH displays the greatest sequence homology to chicken GH of all the GH examined, with 88% of amino acid residues being identical (12), but it has very low lipolytic activity. Many of the differences in amino acid residues between chicken and sea turtle GH are conservative substitutions and, hence, are unlikely to influence biological activity (Fig. 1: residues 27, 40, 43, 44, 81, 92, 95, 100, 110, and 114). The 20-kDa variant (residues 32–45 of human GH deleted; numbered residues 33–46 in Fig. 1 sequence alignment) of human GH retains lipolytic activity (20), confirming that residues 40, 43, and 44 are unlikely to be the reason for the loss of biological activity. Allowing for acceptable substitutions (Tyr ↔ Phe*, Arg ↔ Lys**, Ser ↔ Thr***, Met ↔ Leu****, Ile ↔ Val*****), most amino acid residues (2, 3, 7, 8, 10, 11, 13, 14, 16–18, 21, 22, 24, 25, 28, 29*, 31–33, 35, 41, 42**, 47 [Asn^{47} in sturgeon; H. Kawauchi *et al.*, unpublished observations], 49, 53, 54*, 55, 56, 57***, 58, 59, 61, 62***, 64**, 68–71, 73****, 74–77, 79, 80, 82–87, 89, 91, 93, 96, 97, 99, 101, 102, 103*, 104, 106, 107, 109, 113, 115–120, 123, 124, 127–129, 133, 136, 139**, 141, 142, 144–146, 153–162, 164–166**, 167, 168, 169****, 171–175*, 176, 177**, 178*****, 181, 182, 186, and 189) may be eliminated from contention since they are found in all lipolytically active species (chicken, bovine, human) and in one or more of the nonlipolytic GH species (sea turtle, bullfrog, sturgeon, blue shark, salmon, bonito, yellow tail, or tuna GH; human placental lactogen). At some positions (Fig. 1: residues 9, 50, 52, 88, 130, 135, 137, and 148), there are marked differences between chicken, human, and bovine GH sequences, yet all three retain lipolytic activity. Therefore, these nonconserved residues are less apt to be critical for biological activity.

There is, however, a single substitution that might explain the differences in lipolytic activity between avian, mammalian, and lower vertebrates (particularly with sea turtle GH). A Pro^{132} residue is present in human, bovine, and chicken GH, but is nonconservatively substituted in lipolytically weak/inactive GH species, i.e., sea turtle and salmon (Leu); sturgeon (Phe; H. Kawauchi *et al.*, unpublished observations); bullfrog (Val); blue shark (Ser); and bonito, yellow tail, and tuna (Glu). Extrapolating from x-ray crystallography studies of porcine GH (48), the Pro^{132} residue lies between the third (residues 106–127) and fourth α-helices (residues 152–183). With its cyclic side-chain and low conformational flexibility, proline can have particularly profound effects on the three-dimensional structure of proteins. Proline is generally considered to be a helix-

breaking residue and displays a statistical preference for β-turn conformations, often located at the exposed surface of proteins (49). Therefore, a single amino acid substitution for Pro^{132} could eliminate a β-turn required for receptor/receptor subunit activation (i.e., lipolysis) in lower vertebrate GH.

There are very marked sequence differences between the lower vertebrate GH (blue shark, chum salmon, yellow tail, bonito, tuna, bullfrog, and sea turtle) and those of the homeotherms (chicken, bovine, and human) examined thus far, yet all are active in inhibiting glucagon-induced glycerol release from chicken adipose tissue (Tables III and IV). This would suggest that there is less selectivity for the antilipolytic response than for the lipolytic. As many of these GH also have very low rat tibia test activity (38–40), it appears that the chicken adipose antilipolytic receptor/receptor subunit has less ability to discriminate between different GH structures than the rat tibia receptor/receptor subunit.

Human placental lactogen, being 85% and 54% homologous to human and chicken GH, respectively (Fig. 1), is the only GH homolog examined to date that does not inhibit glucagon-induced lipolysis in chicken adipose tissue (20). Comparing the hPL sequence with antilipolytically active GH species (Fig. 1; counting residue 1 as deleted) reveals several nonconserved residues that could possibly account for the lack of antilipolytic activity: Gln^{3}, Gln^{17}, Met^{64}, Asp^{108}, Asp^{109}, and His^{152}. Of these residues, Gln^{17} would be located in helix-1 and $Asp^{108,109}$ in helix-3, while Gln^{3}, Met^{64}, and His^{152} would lie outside of helical regions (alluding to porcine GH x-ray crystallography data [48]). A larger structure-activity database, including GH homologs that vary at these positions, will be necessary to further elucidate the antilipolytic structural requirements.

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