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Characterization of a Bioactive 15 kDa Fragment Produced by Proteolytic Cleavage of Chicken Growth Hormone

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There is evidence for a cleaved form of GH in the chicken pituitary gland. A 25 kDa band of immunoreactive-(ir-)GH, as well as the 22 kDa monomeric form and some oligomeric forms were observed when purified GH or fresh pituitary extract were subjected to SDS-PAGE under nonreducing conditions. Under reducing conditions, the 25 kDa ir-GH was no longer observed, being replaced by a 15 kDa band, consistent with reduction of the disulfide bridges of the cleaved form. The type of protease involved was investigated using exogenous proteases and monomeric cGH. Cleaved forms of chicken GH were generated by thrombin or collagenase. The site of cleavage was found in position Arg_{133}-Gly_{134} as revealed by sequencing the fragments produced. The NH_2-terminal sequence of 40 amino acid residues in the 15 kDa form was identical to that of the rcGH and analysis of the remaining 7 kDa fragment showed an exact identity with positions 134–140 of cGH structure. The thrombin cleaved GH and the 15 kDa form showed reduced activity (0.8% and 0.5% of GH, respectively) in a radioreceptor assay employing a chicken liver membrane preparation. However, this fragment had a clear bioactivity in an angiogenic bioassay and was capable to inhibit the activity of deiodinase type III in the chicken liver.

Key Words: Growth hormone; proteolytic cleavage; biological activities of GH variants.

Introduction

Multiple forms or variants exist for members of the growth hormone (GH)-prolactin (PRL) hormone family. Post-translational changes are responsible for many of the variants including deamidation, glycosylation, phosphorylation, proteolytic cleavage and, in some cases, reduction (reviewed in refs. *1–3*). This article focuses on proteolytically cleaved variants of GH. Cleaved variants of GH have been observed in human pituitary tissue *(4)*. Endogenous proteolytic cleavage can occur between amino acid residues 134 and 149 resulting in a two-chain structure linked by disulfide bonds (reviewed in refs. *2,4*). A very similar, if not identical, variant can be generated by thrombin treatment of ovine, bovine, and human GH *(5,6)* and, in the case of the human GH, this proteolytically cleaved form retains biological activity *(6,7)* and radioreceptor activity *(8)*. It has been reported that plasmin-cleaved human GH has marked differences in binding characteristics (normal binding to high affinity sites, but very low binding to low affinity sites) in a radioreceptor assay using rabbit liver membranes *(9)*. However, it should be noted that upon reduction, the larger fragment (1–134) of human GH was reported to loose much of its biological activity *(6)*.

Strong evidence exists for a second site of proteolytic cleavage, between amino acid residues 43 and 44. Cleavage at this site would result in two fragments: human $hGH_{1–43}$ and $hGH_{44–191}$. The smaller fragment has been isolated from human pituitary tissue *(10)* and has some biological activity on aspects of carbohydrate metabolism *(11)*. The presence of $hGH_{44–191}$ in the pituitary gland should be a natural corollary to the generation of the $GH_{1–43}$. There is also direct evidence for the presence of the larger fragment *(12)*. Moreover, recombinant $hGH_{44–191}$ has been found to have marked diabetogenic activity *(12)*. Additionally, $hGH_{44–191}$ has been reported in the pituitary and in human blood circulation by radioimmunoassay analysis *(13)*. Serum concentrations of this peptide were higher in pregnant than in nonpregnant women *(13)*. While $hGH_{1–43}$ has no detectable somatogenic activity either in GH radioreceptor assays with rabbit liver membranes or with biosynthetic GHBP, $hGH_{44–191}$ has significant, albeit low, activity (respectively, 9.6 and 1.6% that of GH) in the two systems but is even less active (0.03% that of GH) in stimulating mouse GHR expressing FDC-P1 cells *(14)*. Proteolysis by thyroid tissue has been demonstrated at both cleavage sites *(15)*.

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Considerable attention has also been focused on proteolytic cleavage of rodent prolactin. Cleaved forms of prolactin have been observed in pituitary glands from mice *(16)* and rats *(17)*. Moreover, a cleaved form of prolactin can be generated by incubation with prostate tissue *(18)* and membrane fractions from mammary tissue and liver tissue *(19)*. The cleaved rat prolactin has similar radioreceptor and biological activities to the uncleaved form *(19,20)*. However, reduction of the cleaved form to generate 16 kDa rat prolactin considerably reduces its radioreceptor and lactogenic activity *(20)*. Interestingly the 16 kDa form shows binding to a number of tissues that are poorly inhibited, if at all, by either the cleaved or noncleaved forms of rat PRL *(21)*. This finding suggests a different receptor for the 16 kDa form and different biological activities. The 16 kDa rat prolactin has been demonstrated to inhibit angiogenesis, while the intact form was without effect *(22–24)*. Moreover, there are specific receptors for the 16 kDa form on capillary endothelial cells *(25)*. It should be noted that the rat hypothalamo-neurohypophyseal system releases a 14 kDa prolactin that has biological activities similar to the 16 kDa form *(26,27)*.

The present study examines (a) whether a natural cleaved variant of GH exists in the chicken pituitary gland, (b) whether exogenous proteases can generate a cleaved form of chicken GH, (c) whether a subcellular fraction from chicken liver can cleave chicken GH, (d) whether cleavage and/or reduction influences the biological activity of GH (radioreceptor assay, angiogenesis, and inhibition of deiodinase type III activity), (e) the implementation of a method to produce reasonable amounts of a 15 kDa cGH fragment to characterize it further, and (f) to determine the exact site of cleavage to produce the 15 kDa fragment.

Results

Presence of an Endogenously Cleaved GH in the Chicken Pituitary Gland

To examine whether a naturally occurring cleaved GH is present in the chicken adenohypophysis, a freshly prepared pituitary homogenate was subjected to SDS-PAGE under both nonreducing (NRC) and reducing conditions (RC), and compared with a purified preparation of cGH obtained by immunoaffinity chromatography with a monoclonal antibody. Immunoreactive GH was visualized following Western blotting and immunostaining. Under NRC (Fig. 1A), an ir-band (apparent molecular weight 25 kDa) somewhat larger than monomeric GH (MW 21.5 kDa) was observed irrespective of whether the GH in the pituitary extract or the purified pituitary GH is considered. In addition, MW bands of 42 kDa, 76 kDa, and higher representing dimer, trimer GH, and other oligomeric forms were also observed. Submonomeric forms were also present. This pattern of ir-GH bands has been consistently observed over several years of study.

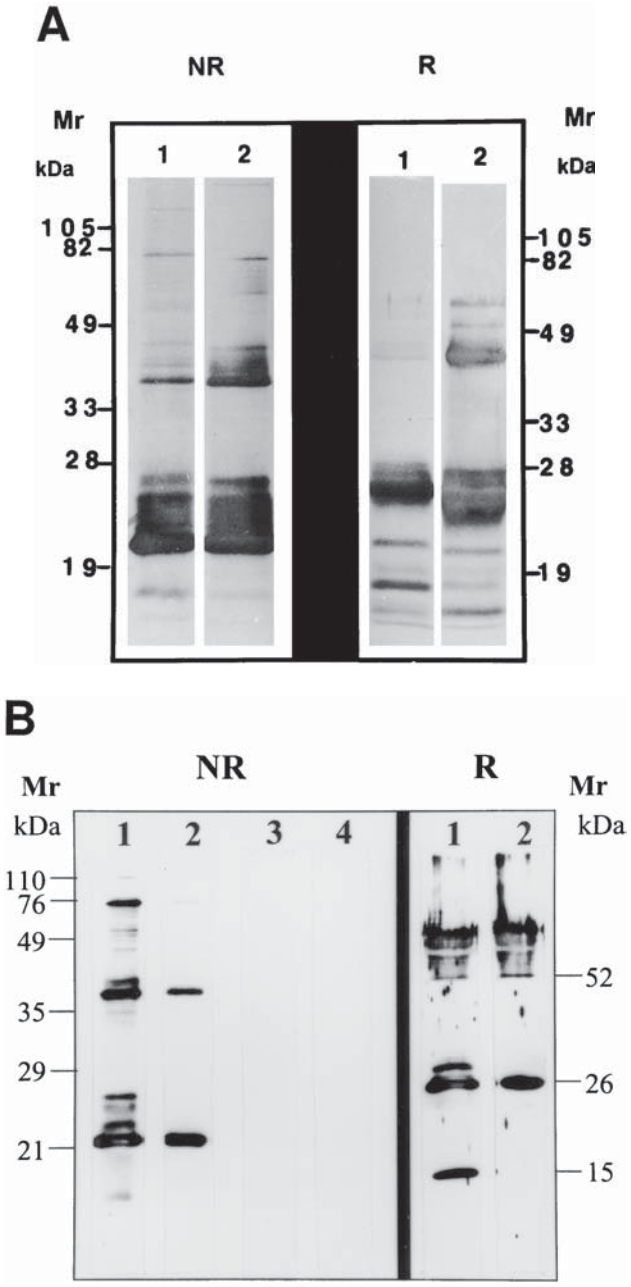

Fig. 1. (**A**) Size variants of chicken GH by SDS-PAGE and western blot. (NR) SDS-electrophoresis under non-reducing conditions. Lane 1: fresh pituitary homogenate (6 μg); lane 2: purified GH (200 ng, obtained by immunoaffinity chromatography). (R) SDS-electrophoresis under reducing conditions. Lane 1: fresh pituitary homogenate (6 μg); lane 2: purified GH (200 ng, obtained by immunoaffinity chromatography). The western blot was developed with diaminobenzidine and H_2O_2 as substrates for HRP. (**B**) Size variants of chicken GH by SDS-PAGE and Western blot. (NR) SDS-electrophoresis under nonreducing conditions. Lane 1: fresh pituitary homogenate (750 ng); lane 2: purified GH (50 ng, B-DE-1); lane 3: fresh pituitary homogenate (750 ng) treated with the first antibody preadsorbed with excess cGH; lane 4: fresh pituitary homogenate (750 ng) in the absence of second antibody (goat anti-rabbit IgG coupled to HRP). (R) SDS-electrophoresis under reducing conditions. Lane 1: fresh pituitary homogenate (750 ng); lane 2: purified GH (50 ng, B-DE-1). The western blot was developed with ECL (Amersham) by chemiluminescence and luminography.

Since the result of Fig. 1A showed a large smear of immunoreactive forms of GH between 20 and 28 kDa in the NRC and there was a smear in a slightly higher molecular weight area under the RC, it was decided to repeat the experiment using smaller amounts of material to avoid a congestion in the mentioned areas. The results are shown in Fig. 1B. It is clear that under NRC the pituitary extract (lane 1) presented several immunoreactive forms (15, 22, 24, 25, 42, 46, 76 kDa). In the area corresponding to the smear in Fig. 1A, this new experiment showed the presence of three main bands (22, 24, 25 kDa). The dimeric and oligomeric forms were also observed. Lane 2 in Fig. 1B shows the result obtained with purified cGH (B-DE-1 as in ref. *42*). Only the monomeric and dimeric forms were observed. Lane 3 shows a control of pituitary extract treated with a first antibody preadsorbed with excess recombinant cGH. It can be appreciated that all the bands present in lane 1 disappeared when the specific antibody was blocked by pure rcGH. Lane 4 shows another control where the second antibody was not added in the Western blot of the pituitary extract and again no bands were visible. This result accounts for the absence of endogenous peroxidase in the extract, which eventually could react unspecifically with the substrate. Under reducing conditions, the pituitary extract (lane 1) showed four bands: the dimer (52 kDa), the monomer (26 kDa), a putatively glycosylated (29 kDa) form, and a submonomeric form (15 kDa). In lane 2 under RC, the pure cGH (B-DE-1) showed only the monomer and the dimer. In both lanes an artifact that often appears under RC is observable at around 65 kDa. There are some important issues to consider for comparing the results of Fig. 1A and Fig. 1B: in the first case, 6 µg of the pituitary extract and 200 ng of purified cGH were analyzed, whereas in the second case 750 ng of pituitary extract and 50 ng of pure cGH were studied. This was due to the sensitivity of the development methods used. In the experiment corresponding to Fig. 1A diaminobenzidine was employed as substrate to the second antibody coupled to horseradish peroxidase, whereas in the experiment corresponding to Fig. 1B the substrate was ECL (Amersham) and was developed by chemiluminescence and luminography. Two different sources of purified cGH were also employed. The cGH used for the experiment in Fig. 1A was obtained by immunoaffinity chromatography employing a monoclonal antibody, which recognizes most of the cGH variants *(55)*. The hormone used for the experiment in Fig. 1B was purified by a more conventional method, which includes $Ca(OH)_2$ and ammonium sulfate precipitation followed by ion-exchange chromatography *(42)* and recovers mainly the monomeric form. With lyophilization, a certain amount can be converted into the dimeric form.

Besides the monomer observed in the pituitary extract in Fig. 1B, two strong immunoreactive bands were present (24 and 25 kDa) under NRC. Of these, one dissapeared when the sample was analyzed under RC giving rise to an increase

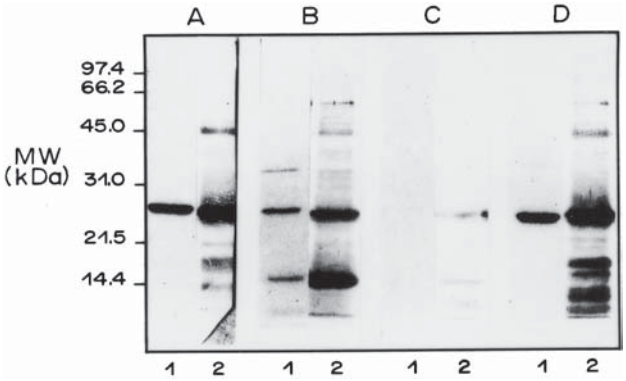

Fig. 2. Effect of thrombin, trypsin, or protease V8 on chicken GH as determined by SDS-PAGE under reducing conditions. (**A**) Chicken GH alone (lane 1: Coomassie blue staining, lane 2: immunostaining after Western blot). (**B**) Chicken GH incubated at 37°C for 2 h with human thrombin (lane 1: Coomassie blue staining, lane 2: immunostaining after Western blot). (**C**) Chicken GH incubated at 37°C for 2 h with trypsin (lane 1: Coomassie blue staining, lane 2: Western blot). (**D**) Chicken GH incubated at 37°C for 2 h with protease V8 (lane 1: Coomassie blue staining, lane 2: Western blot).

in the 15 kDa band that was only slightly present under NRC. By comparing with the results shown in Figs. 3 and 4A, the 25 kDa band observed in Fig. 1B is the candidate for the cleaved form of cGH, whose structure depends on disulfide bridges, and which under reduction produces the 15 kDa fragment. The other band (24 kDa) may correspond to a glycosylated variant of the hormone, which is still present under RC (29 kDa).

Can Exogenous Proteases Cleave Chicken GH?

In an attempt to investigate the site of cleavage and also to generate cleaved GH, purified GH was incubated with proteases of known specificity (trypsin, thrombin, protease V8, and collagenase, respectively). Proteolysis was estimated by SDS-PAGE under reducing conditions, Western blotting, and immunostaining. Following incubation with trypsin, no ir-GH bands were observed (Fig. 2C). This finding is consistent with the complete degradation of the GH. Incubation with protease V8 produced several submonomeric ir-GH bands (Fig. 2D), although digestion was incomplete, since a substantial amount of the original protein was still present when analyzed under RC. However, incubation of GH with thrombin generated a fragment with an apparent mol wt of 15 kDa (Fig. 2B), as found endogenously in the chicken pituitary gland. As thrombin was the only protease tested that generated a single proteolytic cleavage, it was decided to examine this further.

Examination of the known sequence of amino acid residues in chicken GH *(29)* revealed a potential site for thrombin cleavage (Pro_{132}-Arg_{133}-Gly_{134}), which on reduction would generate a fragment of the right size, 15 kDa. The effect of thrombin on GH cleavage was compared to that of collagenase in view of the existence of only a single pro-

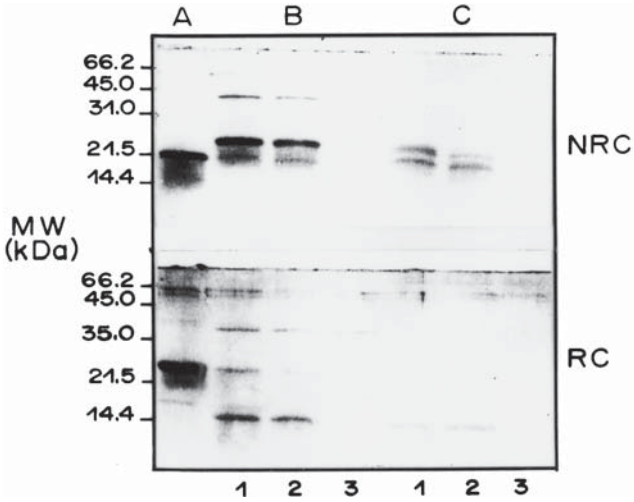

Fig. 3. Effect of thrombin or collagenase on chicken GH as determined by SDS-PAGE under nonreducing conditions (NRC) (upper panel) and reducing conditions (RC) (lower panel). (**A**) Purified chicken GH (lane 0). (**B**) Thrombin incubated with chicken GH at 37°C for 2 h (lane 1), overnight (lane 2) or without GH (lane 3). (**C**) Collagenase incubated with chicken GH at 37°C for 2 h (lane 1), overnight (lane 2), or without GH (lane 3).

teolytic site (Pro_{132}-Arg_{133}-Gly_{134}-Pro_{135}) in the same locus as that for thrombin. It is readily apparent that when the incubated GH is analyzed by SDS-PAGE under NRC, followed by Coomassie blue staining (Fig. 3, upper panel), both thrombin and collagenase reduced the intensity of the 21.5 kDa monomer GH band and, also, that a 25 kDa band was generated. It might be noted that the band at approx 40 kDa in lanes B-1–3 under either NRC or RC is thrombin. If the GH incubated with either protease is then analyzed by SDS-PAGE under RC, the 25 kDa ir-GH band is no longer observed while the 15 kDa ir-GH band is now readily visible with the protease-treated GH. This result is consistent with both thrombin or collagenase being capable of cleaving chicken GH.

Determination of the Cleavage Site to Produce the 15 kDa GH Fragment with Thrombin

In order to produce large amounts of the 15 kDa GH fragment to continue its chemical and biological characterization, rcGH was submitted to thrombin digestion and the proteolytic product was purified by electroelution after SDS-PAGE. Figure 4A shows the results after each step of the procedure, analyzed by SDS-PAGE under NRC and RC, respectively. Again, under nonreducing conditions a band of around 25 kDa (lane 2) was produced after cleaving rcGH with thrombin. When this cleaved form was reduced, a band of 15 kDa was observed (lane 3), and the same mol wt was retained after carbamidomethylation of the reduced product (lane 4). When analyzed under reducing conditions, a band of 15 kDa was observed after cleaving with thrombin, after reducing with DTT, and after carbamidomethylation. Figure 4B shows another experiment where both the 15 kDa and the 7 kDa fragments produced by thrombin digestion were observed by SDS-PAGE (15% separating gel) under RC and Western blotting. After electroelution of both bands, sequence analysis was performed.

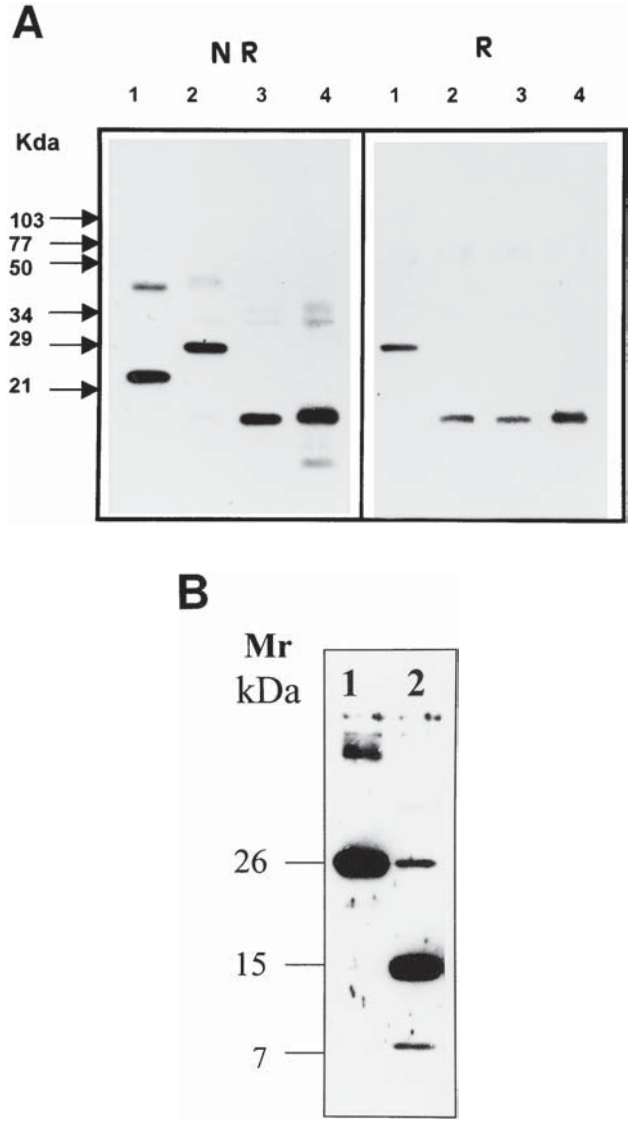

Fig. 4. (**A**) Electrophoretic analysis of the products of recombinant chicken GH after cleavage with thrombin to produce the 15 kDa cGH fragment. (NR): SDS-PAGE under nonreducing conditions and Western blot, lane 1: recombinant cGH alone; lane 2: rcGH after cleavage with thrombin; lane 3: isolated 15 kDa rcGH fragment after electroelution; lane 4: 15 kDa rcGH fragment after carbamidomethylation. (R): SDS-PAGE under reducing conditions and western blot, lane 1: recombinant cGH alone; lane 2: rcGH after cleavage with thrombin; lane 3: isolated 15 kDa rcGH fragment after electroelution; lane 4: 15 kDa rcGH fragment after carbamidomethylation. (**B**) SDS-electrophoresis (15% separating gel) and Western blot analysis of the products of recombinant chicken GH after cleavage with thrombin to produce the 15 kDa and the 7 kDa cGH fragments. Lane 1: recombinant cGH alone; lane 2: rcGH after cleavage with thrombin. The analysis was performed under reducing conditions.

The 15 kDa fragment produced by thrombin treatment of recombinant cGH was purified by semipreparative SDS-PAGE and its NH_2-terminal sequence determined. The sequence was **AFPAMPLSNLFANAVLRAQHLHLLAAET YKEFERTYIPEDQRY** which is identical to the published sequence for NH_2-terminus of chicken GH *(29)* except for the NH_2-terminal alanine.

Also, the small 7 kDa fragment produced by thrombin cleavage was isolated by electroelution and directly sequenced. The $–NH_2$-terminal analysis showed the following sequence: **GPQLLRP**, which corresponds exactly to residues 134–140 in the primary structure of cGH.

Can a Subcellular Fraction of Chicken Liver Cleave cGH?

To examine whether a target tissue for GH could also generate a cleaved GH, purified chicken GH was incubated with subcellular fractions of chicken liver. Incubation for 2 h with the 25,000*g* pellet at 37°C and pH 3.5 (the optimum pH for an acid protease presumed to be present) resulted in complete disappearance of GH as indicated by SDS-electrophoresis. If the incubation time was reduced to 30 min, a cleaved 25 kDa GH (as analyzed by SDS-PAGE under NRC) was generated. This protein on RC ran as a 15 kDa band (data not shown). Incubation of GH at pH 4.5 and 5.5 with the liver subcellular preparation resulted in no apparent change to the GH as analyzed by SDS-PAGE under both RC and NRC. However, incubation at pH 7.5 and 9.5 did result in the appearance of a 15 kDa band. It would therefore appear that a cleaved GH(s) can be generated by liver protease(s).

Biological and Radioreceptor Activity of Cleaved GH and the 15 kDa Fragment Formed by Reduction

A number of attempts were made to isolate the 25 kDa GH (the putative cleaved form) and the 15 kDa GH (the putative fragment generated by reduction of the cleaved form) by electroelution of SDS-PAGE gels using either purified GH or fresh pituitary glands as the source of GH. These methods did not yield significant quantities of either 15 or 25 kDa GH. An alternative approach was employed, in which recombinant chicken GH was incubated with thrombin for 30 min and the resulting mixture subjected to SDS-PAGE under NRC for the 25 kDa GH and under RC for the 15 kDa GH. The 15 and 25 kDa forms of GH were electroluted from the gels and their activities were compared to recombinant and pituitary derived chicken GH in a radioreceptor assay (see Fig. 5).

It is readily apparent that both the 25 and 15 kDa GH had reduced potencies in the radioreceptor assay; these being, respectively, 0.76 and 0.48% that of recombinant GH on a weight basis (3.5 and 2.2% that of pituitary GH). This potency for 15 kDa GH is an overestimate, and should be corrected for the differences in molecular weights. Thus, on a molar basis, 15 kDa GH is 0.33% as potent as recombinant chicken GH and 1.5% that of the pituitary GH. Table 1 summarizes the Scatchard analysis of the radioreceptor assay. Binding of recombinant GH, pituitary GH, and 25 kDa GH can be described based on a single-receptor model. If the Ka and estimated receptor number/concentration are corrected for the potencies of the preparations in the radioreceptor assay, very similar values are observed for the three preparations. However, binding of the 15 kDa GH can

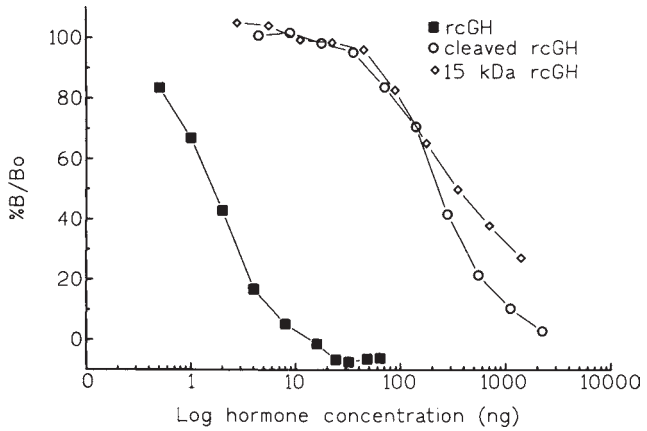

Fig. 5. Displacement of ^{125}I-cGH from chicken liver membrane preparation by 25 kDa (cleaved) and 15 kDa chicken GH. Each point represents the mean of three replicates.

Table 1
Effect of 25 kDa (Cleaved) and 15 kDa Chicken GH (a Fragment Resulting From Reduction of the 25 kDa Form) in a Chicken Liver Membrane Radioreceptor Assay[a] (Using ^{125}I-Recombinant Chicken GH as the Tracer)

	Preparation		
Parameter	RcGH	25 kDa cGH	15kDa cGH
ED_{50} (ng/mL)	1.63	215	342
Potency (ng equiv. rcGH/μg)	1000	7.6	4.8
K_a (L/mol)	1.13×10^{10}	8.19×10^{7}	2.27×10^{7}
K_a (L/mol) corrected for potency	1.13×10^{10}	1.08×10^{10}	0.47×10^{10}
K_a (L/mol) corrected for potency and determined based on a two binding site model	—	—	0.77×10^{10}; 1.67×10^{9}
Number of binding sites (fmol)	12.6	1650	4900
Number of binding sites (fmol) corrected for potency	12.6	12.6	23.3
Number of binding sites (corrected for potency) if calculated based on a two binding site model	—	—	8.9; 60.3

[a] Data calculated by Scatchard analysis.

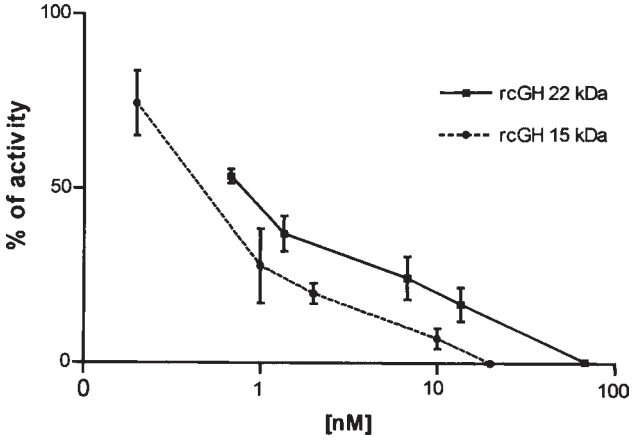

Fig. 6. Effect of recombinant chicken GH and 15 kDa fragment of cGH on the activity of 5 deiodinase type III in chick embryonic liver cells, in vitro during a 24 h incubation.

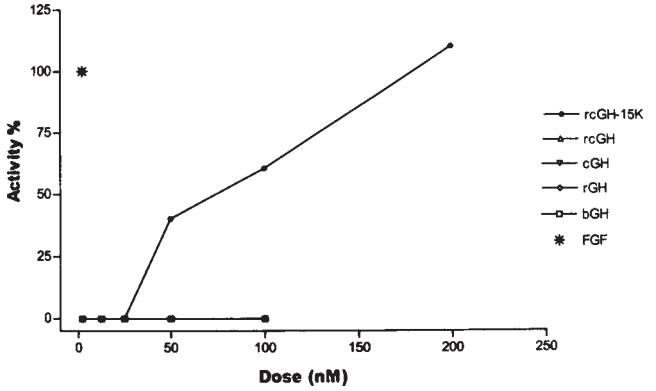

Fig. 7. Effect of recombinant cGH and native GHs (chicken, rat, bovine) and of the 15 kDa fragment of GH on the proliferation of bovine endothelial cells in vitro (data are expressed as the percentage of the proliferation in the presence of bFGF).

best be described by a two binding site model. In this case the binding characteristics (corrected for potency) of the high affinity site are very similar to that with either recombinant or pituitary GH.

Despite the low radioreceptor assay potency for the 15 kDa fragment of GH, its biological activity was examined in different bioassay systems in order to determine if this fragment presented any biological activity. It has been described that growth hormone is capable to depress hepatic 5-deiodinase type III in vitro *(30)*. The 15 kDa cGH fragment was essentially equipotent with recombinant chicken GH in inhibiting the embryonic hepatic 5-deiodinase type III activity in vitro (Fig. 6), showing a clear dose-dependent effect. Also, in view of the profound effects of a 16 kDa fragment of prolactin on angiogenesis (see Introduction for references), the effects of GH and the 15 kDa fragment on endothelial cell proliferation were compared. While neither recombinant chicken GH nor any of the purified native chicken, bovine, or rat GHs exhibited any angiogenic activity, the 15 kDa fragment greatly enhanced endothelial proliferation in a dose dependent manner (Fig. 7). The maximal responses to the fragment and the positive control bFGF were similar.

Discussion

There is increasing evidence that GH, prolactin, and placental lactogen exist in a variety of chemical forms or variants. The biological significance of this phenomenon is still not fully clear. Some variants may represent purification/extraction artifacts or intermediates in hormone synthesis/processing/packaging or degradation. For any variant to be biologically significant as a hormone, it should be released and be biologically active. Moreover, its importance will be enhanced if its secretion and/or clearance is under differential control from other variants, and if it exhibits differences in the spectrum of biological activities compared to other variants. In the case of glycosylated prolactin, there is evidence that at least some of these criteria are met. There are differences in (a) control with cAMP being less effective in inducing secretion in vitro *(31)*; (b) synthesis/secretion with shifts in the ratio of glycosylated to nonglycosylated prolactin in pigs during ontogeny *(32)*, pregnancy/lactation *(33)*, and stress *(34)*, as well as in turkey hens during a reproductive cycle *(35)*; and (c) clearance rate *(36)* and biological activities *(37,38)*.

The situation with cleaved GH or prolactin is perhaps less clear cut. Cleaved prolactin and GH have been observed in the pituitary, including the present studies with GH [prolactin *(20)* GH, reviewed in refs. *1,2*]. The cleaved hormones are frequently viewed as very similar in biological activity to the uncleaved monomeric forms (reviewed in ref. *2*). However, there are reports that cleavage products of human GH either have greatly enhanced activities *(8)* or reduced affinity for one binding site *(9)*. Reduction of the cleaved form of either human GH or rat prolactin reduces somatogenic and/or lactogenic activities *(6,20)*. However, at least in the case of the 16 kDa rat prolactin variant has different but specific binding sites *(20,21)*, and exhibits a biological effect not seen with intact prolactin, that being inhibition of angiogenesis *(22)*. The present studies provide evidence that a 15 kDa fragment of GH has angiogenic activity (Fig. 7). This may represent the fragment antagonizing any endogenous 16 kDa prolactin, which has been recently found to be produced by bovine endothelial cells *(39)*.

There is strong evidence that chicken GH exists as a series of chemically distinct variants that presumably result by post-translational modification *(40–45)*. These include a glycosylated GH *(28,40)* and phosphorylated GH *(43,45)*. The present work provides evidence for the existence in the

chicken pituitary gland of a naturally cleaved GH (which on reduction yields a 15 kDa immunoreactive fragment) (Figs. 1A and 1B). This result is consistent with the situation in the human (e.g., *43*), but has not received attention in other mammalian species or in other classes of vertebrates. The present data on the existence of a cleaved GH in the chicken pituitary gland do not agree with a previous report from the same species *(41)*. It may be argued that the cleaved GH observed (Fig. 1) represents an artifact of the harsh extraction procedures for the purification of GH *(44)*. However, cleaved GH was observed in immunoaffinity purified GH and in freshly homogenized pituitary tissue. Moreover, protease inhibitors (PMSF and aprotinin) were present in both the GH isolation and in the homogenization procedure. It is possible that the cleaved form of chicken GH was missed previously, due either to the presumption that it was an artifact or to age or strain differences in the pituitary tissue used. Indeed there are differences in the relative proportion of cleaved to noncleaved and also cleaved to reduced and cleaved GH in the chicken pituitary gland during growth and development *(46)*. The absence of a 25 kDa band in the preparation corresponding to purified cGH (B-DE-1) in Fig. 1B is explained by the fact that the purification method employed recovers mainly the monomeric form of the hormone, as has been shown before *(42)*.

The putative site of proteolytic cleavage of chicken GH, in the pituitary gland and by the liver, can be inferred based on the molecular size of the reduced cleaved form (15 kDa) on SDS-electrophoresis. The size of the 15 kDa form is consistent with a single cleavage somewhere between amino acid residues 130–140 or 45–55. A potential cleavage site in chicken GH exists between residues 133 and 134 (NH_2–Arg_{130}-Ser_{131}-Pro_{132}-Arg_{133}-Gly_{134}-Pro_{135}-Glu_{136}-Leu_{137}-Gly_{138}-Arg_{139}-Pro_{140}–COOH) which is susceptible to thrombin (consensus site -Pro-Arg-Gly-), collagenase (consensus site -Pro-X-Gly-Pro-), and presumably pituitary protease(s). This site is also proposed as a potential cleavage site by thrombin for human GH *(5)*, since substitution of Arg_{134} and Thr_{135}, with Asp_{134} and Pro_{135} yielded a thrombin-resistant hGH mutant, and substitution of Arg_{134}, Thr_{135}, and Lys_{140} with Asp_{134}, Pro_{135}, and Ala_{140} yielded a plasmin–resistant mutant. This latter mutant was also insensitive to in vitro proteolysis by human plasma incubated for 7 d. These alterations in hGH sequence did not disrupt its biological conformation and retained full growth-promoting activities on rat Nb2 cells and human T-47D breast cancer cells *(47)*. N-terminal sequence analysis of the 15 kDa cGH fragment provides further evidence that the cleavage is not close to the N-terminus. Furthermore, after isolating the small fragment (7 kDa) also produced by thrombin cleavage of chicken GH, it was submitted to –NH_2-terminal analysis by automatic sequencing. The sequence obtained (GPQLLRP) corresponds exactly with residues 134–140 in the cGH primary structure, thus confirming that the site of cleavage by thrombin is Arg_{133}-Gly_{134}. The ability of liver subcellular fractions even at neutral pH to cleave chicken GH suggests that the proportion of cleaved chicken GH in the circulation, or perhaps locally in the target tissue, may be higher than in the pituitary gland. However, in view of the ability of thrombin to cleave GH, caution should be expressed in detecting cleaved GH in serum samples or in plasma when clotting has begun.

In the present study, a radioreceptor assay using chicken liver membranes was employed to examine the activities of the 15 and 25 kDa forms of ir-GH. In this system, a K_a of 1.13×10^{10} L/mol was calculated for rcGH (Table 1). This value is in strong agreement with the value (K_a 1.03×10^{10} L/mol) reported by Krishnan et al. *(48)* with a very similar assay. Reduced radioreceptor activity was observed with 25 kDa GH, and to a greater extent with the 15 kDa GH. The low potency of the 15 kDa GH is in good agreement with the weak activity of recombinant bovine $GH_{1–132}$ (1.5% that of recombinant chicken GH) observed in a similar system *(49)*; bovine $GH_{1–132}$ being analogous to the expected structure of 15 kDa GH. Similarly, the 16 kDa form of rat prolactin, which may be analogous to the 15 kDa GH, has very low activity in prolactin radioreceptor assays *(20,21)*. Based on the use of a two-site model for binding of the 15 kDa GH, a second receptor analogous to the 16 kDa prolactin binding site *(21)* might be tentatively postulated. Such a receptor could be of physiologic significance in view of the presence of relatively high levels of the 15 kDa GH in the pituitary gland of the chick embryo, but lower levels in post-natal development *(46)*.

The low activity of the 25 kDa cGH differs from the situation with cleaved human GH (reviewed in ref. *2*). However, much reduced radioreceptor potencies are observed for a two chain GH (bovine $GH_{1–66,\ 153–191}$) and for mutant forms of GH in which cysteines were replaced by serine residues *(49)*; pointing to critical structural requirements for binding to the receptor. Moreover, a cleaved human GH (Da1) (d 1) showed no activity in stimulating lipolysis from chicken adipose tissue in vitro (C. G. Scanes and R. M. Campbell, unpublished data). In view of the low radioreceptor activities of the 15 and 25 kDa GH, despite their retaining substantial immunoreactivity, if these forms were found in the pituitary and/or plasma, an overestimation of biologically active GH by radioimmunoassay would result *(50)*. However, it should be noted that the 15 kDa fragment does exhibit strong activity in the two GH biological assays tested, namely, inhibition of 5-monodeiodinase in embryonic chicken hepatocyte cultures (Fig. 6) and stimulation of proliferation of bovine endothelial cells in vitro (Fig. 7).

In the chicken embryo, there is a negative relationship between increasing GH levels and decreasing type III activity from the late embryonic period on. This relationship indicates an inhibitory effect of GH on the 5-deiodination of T3 and T4 *(30,51)*, both at the level of hepatic 5DIII gene

expression and translation of the protein *(52)*. This effect was replicated by the 15 kDa cGH fragment despite the low activity shown for binding to the conventional GH receptor in chicken liver membranes.

Similarly, the 15 kDa cGH fragment was able to stimulate ^{3}H-thimidine incorporation into bovine brain endothelial cells in a dose-dependent manner, in an angiogenic bioassay. This effect was not shared with the intact GHs tested in the same assay (rcGH, cGH, bGH, and rGH), thus suggesting a differential effect in respect to the native hormones. This is a similar finding, although with an opposite action, to what is observed with prolactin and 16 kDa prolactin in this angiogenic bioassay *(23)*. It is possible that this fragment accounts for the angiogenic activity of cGH reported earlier when chicken pituitary gland grafts or pure GH effects were determined on the chorioallantoid membrane *(53)*. In this regard, it is also interesting that the highest proportion of 15 kDa cGH fragment in the chicken pituitary occurs during embryonic development *(46)*. Recently, it was shown that the native members of the human prolactin/growth hormone family are angiogenic, whereas their respective 16 kDa N-terminal fragments are antiangiogenic. Apparently, the opposite actions are regulated in part via activation or inhibition of mitogen-activated protein kinase signaling pathway. In addition, the N-terminal fragments stimulate expression of type I plasminogen activator inhibitor, whereas the intact molecules have no effect, an observation consistent with the fragments acting via separate receptors *(54)*.

In summary, we have shown that cGH can be cleaved to produce a 15 kDa fragment, corresponding to the N-terminus, which is not recognized by the conventional liver GH receptor, but is able to exert different bioactivities. This fragment is produced in vitro by thrombin or collagenase by cleaving in position 133–134. This fragment is also present in the chicken pituitary, and its relative proportion varies during ontogeny, being higher during late embryogenesis *(46)*. Data suggest that it might exert its biological effects through a separate receptor.

Materials and Methods

Biological Material

Preparation of the pituitary extracts: Anterior pituitary glands were collected from 8-wk-old male broilers (at Pilgrim's Pride of México, S.A. C.V. Querétaro, Qro.). Glands were removed within seconds after decapitation and extracts were prepared by homogenizing the tissue in a protease inhibitor solution (0.5 m*M* PMSF and 50 kIU/mL aprotinin, pH 7.2) at a final concentration of 0.3 mg/mL. The supernatant was used as the sample for electrophoretic studies. Alternatively, the extract employed in Fig. 1B was obtained in the presence of a protease inhibitor cocktail (Complete Mini, Roche), which inhibits a broad spectrum of proteases and 0.5 m*M* PMSF.

Purification and N-Terminal Amino Acid Sequence Analysis of Chicken Growth Hormone and Cleaved Forms

Some studies used purified pituitary chicken GH, obtained by two different methods. In one preparation (B-DE-1) cGH was isolated from chicken pituitary glands as described previously *(42,50)*. Alternatively, cGH was purified by immunoaffinity chromatography, following the method described by Berghman et al. *(55)*. The 15 and 25 kDa GH forms were isolated by semipreparative SDS-PAGE (respectively under RC and NRC) following thrombin treatment of recombinant chicken GH. Briefly, a typical experiment consisted in incubation of 1 mg rcGH with 40 µg human thrombin (25:1 w/w) dissolved in 0.1 *M* NH_4HCO_3, pH 8.4, at 37°C over 4 h. After cleavage, the sample was separated in a semipreparative SDS-PAGE (15% separating gel), and after briefly staining a control lane with Coomassie blue, the rest of the gel was frozen and then cut by aligning with the stained gel piece. The gel slices containing the protein of interest were electroeluted in a Bio-Rad chamber at 40 mA for 6 h, at 4°C. After extensive dialysis of the eluate (1 mL against 1 L, 5–6×), it was washed with water (10 mL, 5×) and concentrated using centriprep3 cartridges and then a speed-vac. The 15 kDa cGH was then carbamidomethylated *(56)* to avoid disulfide linkage formation, and thus possible covalent aggregation of the peptide. The carbamidomethylated peptide was desalted by chromatography in a Sephadex G25-80 column. NH_2-terminal amino acid sequence analysis of the cleaved and reduced GH produced by thrombin digestion of recombinant chicken GH was performed using a Procise Protein Sequencer 491 (Applied Biosystems). In a similar form, the 7 kDa fragment produced by the proteolytic cleavage of the hormone was isolated by electroelution and directly submitted to the sequencer.

Electrophoresis, Western Blotting, and Immunostaining

Samples were analyzed by one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 1.0-mm-thick, 6-cm-long slab gels using the buffer system of Laemmli *(57)* in the Mini-Protean II cell (Bio-Rad, Richmond, CA). The acrylamide concentration was 12% in the separating gel and 4% in the stacking gel. The samples were run under reducing (RC) (in the presence of 5% 2-mercaptoethanol) and non-reducing (NRC) conditions. After electrophoresis, the gels were electrotransferred onto Immobilon (Millipore) membranes in 25 m*M* Tris, 192 mM Glycine, 20% methanol at 200 mA during 45 min. The membranes were immunostained with a polyclonal antibody against chicken GH after blocking with 3% bovine serum albumin (BSA) (Sigma, St. Louis) in 10 m*M* phosphate-buffered saline (PBS), pH 7.6, and then incubated with a secondary antibody (goat anti-rabbit IgG coupled to horseradish peroxidase). Development of the membranes was done alternatively by two methods: (1) a colorimetric assay

employing 0.025% (w/v) diaminobenzidine and 0.02% (v/v) H_2O_2 as substrates; or (2) a chemiluminescent assay employing ECL (Amersham) as substrate.

Enzymatic Digestions

Pituitary derived (obtained as above) or recombinant (kindly donated by American Cyanamid, Princeton, NJ) chicken GH were incubated in the presence of different proteases to study the corresponding in vitro cleavage products. The following enzymes were prepared at a concentration of 0.2 µg/µL: (a) trypsin (Sigma, St. Louis, MO) was initially dissolved in 1 m*M* HCl, and then it was diluted for the incubation in 8 m*M* Tris, 10 m*M* $CaCl_2$, 1% glycerol, pH 8.0; (b) protease V8 (Sigma, St. Louis, MO) was dissolved in 10 m*M* PBS, pH 7.8; (c) thrombin (from human plasma, Sigma, St. Louis, MO) in 0.1 *M* ammonium bicarbonate, pH 8.0; d) collagenase (Worthington Biochemical Corp., Freehold, NJ) in 0.1 *M* Tris, 0.01 *M* $CaCl_2$, pH 7.5. The incubation mixture (20 µL) consisted of 3 µL of the hormone solution (2 µg/µL), 15.5 µL of the corresponding buffer and 1.5 µL of the corresponding enzyme solution. The samples were incubated with shaking for 2 h or overnight at 37ºC. At the end of each period, the incubation mixtures were then mixed with electrophoresis sample buffer, boiled and submitted to SDS-PAGE.

Subcellular fractions were prepared from chicken livers for hormone cleavage studies. Tissue was obtained from 10-wk-old white Leghorn hens. It was rapidly removed, rinsed in cold PBS, weighed and immediately frozen on dry ice. A 25,000*g* fraction containing a mixture of mitochondrial and lysosomal enzyme activities was prepared from the frozen livers by the method of Compton and Witorsch *(18)*. The 25,000*g* liver pellet was dissolved in 0.1 *M* Tris-HCl, pH 7.4 and diluted to 8 mg/mL based on the protein content as determined by Bio-Rad protein assay kit with BSA as the protein standard. The membrane preparation was incubated with chicken GH for 30–240 min at 37ºC at various pHs [in 10 µL of 0.1 *M* Tris-HCl, pH 7.4 and 40 µL of corresponding reaction buffer (a) 0.1 *M* citrate-phosphate, 0.15 *M* NaCl, pH 3.5; (b) 0.1 *M* citrate, 0.15 *M* NaCl, pH 4.5; (c) 0.1 *M* citrate, 0.15 *M* NaCl, pH 5.5; (d) 0.1 *M* Tris-HCl, pH 7.4; (e) 0.1 *M* Tris-HCl, pH 9.5]. Heat-inactivated (92ºC, 15 min) pellets of the subcellular preparations were used as the negative controls. At the end of the incubation period, the GH was separated from the pellet by centrifugation at 13,700*g* at 4ºC. The supernatants containing GH were then submitted to SDS-PAGE.

Radioreceptor and Biological Assays

Radioreceptor activity of the 15 and 25 kDa forms of GH was estimated using the system developed by Krishnan and colleagues *(48)*. The tracer, recombinant chicken GH, was iodinated by the iodogen method *(59)*. A liver membrane preparation from adult male chickens (strain white Leghorn) was employed for the binding studies. Either recombinant or pituitary chicken GH were used as the standards.

In addition, biological activities of recombinant chicken GH were compared with cleaved and reduced GH by either the ability to inhibit the activity of deiodinase type III from embryonic chick livers or by the ability to stimulate the proliferation of bovine endothelial cells in vitro. The former system shows good activity of chicken GH and of glycosylated chicken GH *(60)*, while the latter system has extensively been employed with 16 kDa prolactin *(24,25,39)*. Deiodinase type III was determined using liver tissue from 19-d-old chick embryos by the method of Valverde-R and colleagues *(61)*. Briefly, this method entailed incubation of liver homogenate (80 µg protein) with 2 n*M* ^{125}I-T3 and 40 m*M* DTT for 1 h. Bovine brain capillary endothelial cells, isolated by the method of Gospodarowicz and colleagues *(62)*, were kindly donated by Dr. C. Clapp. The cells were grown by the method of Ferrara et al. *(22)* and serially passaged in low-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 2 m*M* glutamine, and penicillin/streptomycin (50 U/mL). Endothelial proliferation was determined by ^{3}H- thymidine uptake.

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