A missense mutation in the GHR gene of Cornell sex-linked dwarf chickens does not abolish serum GH binding

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Abstract

Sex-linked dwarfism (SLD) in chickens is characterized by impaired growth despite normal or supranormal plasma growth hormone (GH) levels. This resistance to GH action is thought to be due to mutations of the GH receptor (GHR) gene that reduce or prevent GH binding to target sites. The genetic lesion causing GH resistance in Cornell SLD chickens is, however, not known. Previous studies have shown that hepatic GH-binding activity is abnormally low in these birds, yet the GHR gene is transcribed into a transcript of appropriate size and abundance. Point mutations or defects in translation could therefore account for the impaired GHR activity in this strain. These possibilities were addressed in the present study.

A missense mutation resulting in the substitution of serine for the conserved phenylalanine was identified in the region of the GHR cDNA encoding the extracellular domain. Translation of this mutant transcript was indicated by the presence of GHR/GH-binding protein (GHBP)-immunoreactive proteins in liver (55, 70 and 100 kDa) and serum (70 kDa) of normal (K) and SLD birds. Radio-labelled GH did not, however, bind to the hepatic membranes of most SLD chickens. Serum GH-binding activity, in contrast, was readily detectable, although at significantly lower levels than in normal birds. The missense mutation in the SLD GHR gene may thus affect targeting of GHRs to hepatic plasma membranes.

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Introduction

Sex-linked dwarfism (SLD) in chickens, like the Laron syndrome (LS) in humans, is an inherited disorder characterized by reduced body weight and longitudinal bone growth, despite normal levels of circulating GH (Guillaume 1976, Decuypere *et al.* 1991, Laron 1993, Rosenfeld *et al.* 1994). Both syndromes may thus result from GH resistance, especially as exogenous GH does not induce metabolic or somatotrophic changes in SLD birds or LS patients (Decuypere *et al.* 1991, Rosenfeld *et al.* 1994). Indeed, deficiencies in hepatic GH-binding activity and corresponding GH receptor (GHR) gene mutations have been detected in numerous SLD and LS populations (Leung *et al.* 1987a, Burnside *et al.* 1991, Parks *et al.* 1997, Sobrier *et al.* 1997). It is now known that both syndromes are highly heterogeneous, resulting from partial gene deletions or from point mutations in splice or stop sites that impair gene expression or from point mutations that cause structural problems in ligand binding, receptor dimerization, or signal transduction (Burnside *et al.* 1991, 1992, Huang *et al.* 1993, Agarwal *et al.* 1994, Cogburn *et al.* 1997, Parks *et al.* 1997).

SLD is, however, also associated with normal transcription of the GHR gene (Hull *et al.* 1992). Studies in the Cornell strain of SLD birds have, for instance, revealed multiple GHR gene transcripts of 4.4, 2.8 and 1.0 kb in normally growing (K) and SLD chickens, and sequencing of most of the coding domain failed to reveal any point mutation or deletion (Hull *et al.* 1992). Defects in GHR translation or post-translational processing may therefore account for GH resistance in these birds. This possibility was therefore examined by determining if hepatic GHR immunoreactivity was present in Cornell SLD chickens. Point mutations or deletions could also be present outside the previously sequenced regions, thus the entire coding region of the SLD GHR was also cloned and sequenced.

A truncated form of the GHR, comprising its extracellular domain, also exists in a soluble form in serum and tissues (Harvey & Hull 1995a). In rodents, this GH-binding protein (GHBP) is synthesized from a splice variant of the GHR gene that replaces the intracellular and transmembrane domains with a short hydrophilic tail (Baumbach *et al.* 1989, Smith *et al.* 1989). In contrast, GHBP in humans, rabbits and chickens are thought to originate from proteolytic cleavage of hepatic GHRs, as an

alternative GHBP-encoding transcript has not been identified in species other than rodents (Spencer *et al.* 1988, Sotiropoulos *et al.* 1991, Cogburn *et al.* 1997). GHRs and GHBP should thus be coordinately impaired in pathophysiological and physiological states of GH resistance, as the two proteins are structurally related and arise from the same gene transcript. Indeed, GHBP are deficient in most, but not all, LS populations (Parks *et al.* 1997) and in GH-resistant fasting states (Harvey & Hull 1995b). The possibility that GHBP may be deficient in SLD chickens was therefore assessed.

Materials and Methods

GHR cDNA cloning and sequencing

Hepatic RNA was isolated from Cornell (K) and SLD birds and subjected to reverse transcription-PCR (RT-PCR) using Taq polymerase, as previously described (Hull *et al.* 1993). Two overlapping fragments of the GHR cDNA, spanning the entire coding region, were amplified. These fragments were subsequently inserted into a commercial vector (pCR 2.1; Invitrogen, Carlsbad, CA, USA) and sequenced by the dideoxy chain termination method (Oncor Sequencing Kit; Fisher Scientific, Edmonton, AB, Canada). RT-PCR was performed on hepatic RNA isolated from at least three different birds of each strain, and three to six cloned PCR products were sequenced from each reaction to ensure that variations from the published GHR cDNA sequence were not due to replication errors.

Western blot analysis

The possibility that SLD serum and liver may be deficient in immunoreactive GHBP/GHR or contain GHBP or GHRs of anomalous size was evaluated by Western blot analysis, as previously described (Hull *et al.* 1996a). GHR/GHBP-like proteins in hepatic homogenates and serum aliquots were detected by a rabbit polyclonal antiserum (α cGHBP at a final dilution of 1:2000) (Hull *et al.* 1996a). For comparative purposes, another polyclonal antibody (α CH17) raised in mice against a putative membrane-bound chicken GHBP (Dr W Baumbach, American Cyanamid, Princeton, NJ, USA) was also used. This protein was expressed by a chicken GHR cDNA clone obtained from an avian hepatic library and contains the extracellular and transmembrane domains of the GHR and a portion of the intracellular domain (Bingham *et al.* 1994). It is, however, uncertain if this protein is expressed *in vivo*, although the existence of tissue GHR/GHBP isoforms is well established (Dastot *et al.* 1996). Replicate blots were incubated in the absence of primary antibody or with antibody that had been preabsorbed with an excess of recombinant CH17 for 1 h. Immunoreactive proteins were

visualized by enhanced chemiluminescence (Amersham, Mississauga, Ontario, Canada).

Radioligand binding

The binding of GH to the hepatic membranes of adult SLD and K birds was determined as described elsewhere (Fraser *et al.* 1990). The livers from 20 chickens of each strain were collected and hepatic plasma membranes were isolated by centrifugation and ultracentrifugation, after homogenization in 25 mmol/l Tris-HCl buffer, pH 7.4. The membranes were then incubated at room temperature for 4 h with 125 I-labelled ovine GH (60 μ Ci/ μ g; oGH-14; NIADDK, Bethesda, MD, USA) in the presence or absence of unlabelled GH (2 μ g/ml), to determine non-specific binding. Bound and free radioactivity were separated by centrifugation. Radioligand-binding sites in SLD and K serum were similarly assessed (Hull *et al.* 1992). Bound and free radioactivity were separated by charcoal adsorption (Hull *et al.* 1996b) or by immunoprecipitation (Fraser *et al.* 1990) using mAb 263 (Agen Biomedical Ltd, Brisbane, Queensland, Australia), a monoclonal antibody raised against the conserved binding domain in mammalian GHBP (Barnard & Waters 1985). Serum GH-binding activity was further assessed by gel filtration on Sephadex G100 (Hull *et al.* 1992), after the incubation of 1 ml of serum with approximately 1.3×10^6 c.p.m. 125 I-labelled (specific activity 40–50 Ci/ μ g) oGH (oGH-14, biological grade, 1.4 IU/mg), in the presence or absence of excess unlabelled ovine GH (Fraser *et al.* 1990). Fractions (1 ml) were collected and aliquots (100 μ l) were counted for radioactivity. The radioactive peaks were characterized by comparison with 125 I-labelled oGH and Na 125 I and with the elution of molecular mass markers (Sigma Chemical Company, St Louis, MO, USA) which were monitored spectrophotometrically at 280 nm. Significant differences in the amount of tracer bound were determined by Student's *t*-test.

Results

GHR cDNA sequencing

GHR cDNA fragments were amplified from reverse-transcribed hepatic RNA from K and SLD birds and sequenced. The nucleotide sequence of the K strain GHR cDNA was identical with that published by Burnside *et al.* (1991) for the GHR cDNA in broiler fowl liver, with the exception of the replacement of 2 G residues with C residues at positions 186–187 (data not shown). This polymorphism was identical with that previously reported by Duriez *et al.* (1993) for the GHR in another Leghorn strain. This polymorphism would result in the substitution of asparagine and leucine for lysine and valine in the extracellular domain of the receptor.

Figure 1 Partial hepatic GHR cDNA sequence from normally growing (K) and sex-linked dwarf (SLD) birds, showing the T-to-C point mutation in the SLD GHR cDNA. GHR cDNAs were amplified by RT-PCR from hepatic RNA, inserted into the pCR 2.1 plasmid, and sequenced by the dideoxy chain termination method.

The sequence of the SLD hepatic GHR cDNA was identical with that in the K strain, with the exception of 1 bp difference in the extracellular GH-binding domain (Fig. 1). This T–C substitution at position 370 would result in a phenylalanine–serine substitution at amino acid 112 (Burnside *et al.* 1991).

Western blotting

The presence of GHBP/GHR immunoreactivity in K and SLD liver and serum was demonstrated by Western blot analysis (Hull *et al.* 1996a). Serum GHBP immunoreactivity was first demonstrated using a polyclonal antibody raised against recombinant chicken GHBP. A 74 kDa protein was labelled in SLD serum which corresponded in size to a protein present in K serum (Fig. 2). A larger protein of 143 kDa was also occasionally detected in the serum of both strains. No labelling was observed if the primary antibody was omitted (data not shown) or replaced with preimmune serum (Fig. 2). Sera were also screened with a polyclonal antibody raised against CH17, but this antibody did not detect any proteins in either K or SLD serum (data not shown). Immunoreactive proteins were of comparable abundance in K and SLD sera.

GHBP-like immunoreactivity was also present in hepatic homogenates (Fig. 3). In both strains, proteins of 55, 70–75, 100–105 and 200 kDa cross-reacted with α GHBP. With the exception of the 200 kDa moiety, the

Figure 2 Western blot analysis showing the cross-reactivity of K (k) and sex-linked dwarf (s) serum proteins with a polyclonal antibody raised against the extracellular domain of the growth hormone receptor (α GHBP). Replicate blots were incubated with a polyclonal antibody raised against a putative membrane-bound GHBP (α CH17). Immunoreactive proteins were visualized using horseradish peroxidase-labelled secondary antibodies and enhanced chemiluminescence. Staining was not observed if the primary antibody was replaced by normal rabbit serum (NRS) (α GHBP) or with α CH17 that had been preabsorbed with recombinant CH17 (p.a. α CH17).

GHBP-like proteins did not cross-react with preimmune rabbit serum (Fig. 3). α CH17 also detected a protein of approximately 55 kDa in K and SLD homogenates, as well as a 64 kDa GHBP-like moiety (Fig. 3). Preabsorption of this antibody with CH17 (Fig. 3) or replacement with preimmune serum (data not shown) totally abolished labelling to the 55 kDa and diminished (by >80%) the labelling of the 64 kDa protein.

Radioligand binding

Plasma membranes from the livers of K birds specifically bound 20% of the 125 I-oGH tracer (Fig. 4). In contrast, very little tracer (<2%) was specifically bound by SLD hepatic membranes ($P<0.001$, in comparison with the

Figure 3 Western blot analysis showing the cross-reactivity of K (K) and sex-linked dwarf (S) hepatic proteins with a polyclonal antibody raised against the extracellular domain of GHR (α GHBP). Replicate blots were incubated with a polyclonal antibody raised against a putative membrane-bound GHBP (α CH17). Immunoreactive proteins were visualized using horseradish peroxidase-labelled secondary antibodies and enhanced chemiluminescence. GHR/GHBP-like proteins were not detected when the primary antibody was replaced by normal rabbit serum (NRS) or was preincubated with recombinant CH17 before Western blot analysis.

Figure 4 GH-binding activity in hepatic membranes from normal (K) and sex-linked dwarf (SLD) birds. Purified plasma membranes were incubated with ¹²⁵I-labelled ovine GH in the presence or absence of unlabelled GH (2 µg/ml), to determine non-specific binding. Bound and free radioactivity were separated by centrifugation. *** $P < 0.001$ (Student's *t*-test). Means $\pm$ S.E.M. ($n=20$).

controls). Indeed, the tracer only bound to the hepatic membranes of two SLD chickens, and at much lower levels (<10%) than observed with membranes from K birds. GH-binding activity was undetectable in 18 other SLD livers.

Although oGH readily bound to the hepatic membranes of K birds, only 3% was bound by their serum proteins, as determined by mAb 263 immunoprecipitation or charcoal adsorption (Fig. 5). GH binding to serum proteins was demonstrable in the sera of all SLDs, but was consistently 30–40% ($P < 0.001$) of that in the controls. The binding of oGH in SLD serum, as in normal Leghorns (Hull *et al.* 1992), was to a single protein 46–52 kDa in size and was reduced in the presence of excess unlabelled GH (Figs 6 and 7).

Discussion

These results demonstrate that a missense mutation in the region of the GHR gene encoding the extracellular domain of the receptor may be causal in the GH resistance in the Cornell strain of SLDs. Indeed, the conservation of this phenylalanine residue in humans, chickens, rabbits and rats (Burnside *et al.* 1991) suggests it is important for GHR function. This mutation has not, however, been identified in any human population (Amselem *et al.* 1996), nor has the importance of this residue been investigated in the human GHR by site-directed mutagenesis (Bass *et al.* 1991). Neighbouring residues are, however, crucial for GHR function, as illustrated in LS cohorts (Amselem *et al.* 1996) and as shown by site-directed mutagenesis (Bass

Figure 5 GH-binding activity in plasma from normal (K) and sex-linked dwarf (SLD) birds. Aliquots of plasma were incubated with ¹²⁵I-labelled oGH for 4 h, and bound and free radioactivity were separated by charcoal adsorption or by immunoprecipitation with mAb 263. ** $P < 0.001$ (Student's *t*-test). Means $\pm$ S.E.M. ($n=4-8$).

Figure 6 Typical elution profile of ¹²⁵I-labelled ovine GH from a 1 $\times$ 50 cm column of Sephadex G-100 after a 4 h incubation of the tracer with plasma from sex-linked dwarf (SLD; $\blacktriangle$) or normal (K; $\blacksquare$) chickens. The elution profile of radiolabelled (*) GH is also shown ($\bullet$).

et al. 1991). For instance, the upstream cysteine residue forms a disulphide bond, whereas the downstream serine residue is necessary for normal protein expression (Bass

Figure 7 Typical elution profile of ^{125}I -labelled oGH from a 1×50 cm column of Sephadex G-100 after a 4 h incubation of the tracer with plasma from SLD chickens in the presence (■) or absence (●) of excess unlabelled GH.

et al. 1991). Site-directed mutagenesis of other residues in the same β -strand as the mutated phenylalanine residue similarly reduces or abolishes GH-binding affinity (Bass *et al.* 1991).

The missense mutation in the SLD GHR gene does not alter transcription or translation of the GHR gene into the GHR and the soluble GH-binding protein, as GHR/GHBP-immunoreactive proteins of equal size and abundance are present in liver homogenates from K and SLD birds. The 100 kDa proteins detected in hepatic homogenates probably reflect the full-length receptor (Agarwal *et al.* 1994), whereas the smaller 55 kDa and/or 70 kDa proteins correspond to soluble GHBPs. Earlier studies employing gel chromatography resulted in different estimates for the size of the GHR and GHBP, as 60–65 kDa GH-binding moieties were detected in hepatic plasma and nuclear membranes, and smaller 55 kDa moieties were observed in hepatic cytosol (Hull *et al.* 1992). The molecular mass of the circulating GHBP as determined by gel chromatography (47–52 kDa: present study and Hull *et al.* 1992) similarly differed from that determined by Western blotting (70 kDa). This size discrepancy may reflect the labile nature of the GHR and GHBP and the extended incubation time required for chromatographic analysis. Indeed, Vasilatos-Younken *et al.* (1991) reported an increase in smaller GHBP-immunoreactive forms (52 kDa) at the expense of the larger 70 kDa proteins after serum storage. A brief report by Janssens *et al.* (1994) also indicated the presence of a 70 kDa GHBP in the serum of different strains of normal and SLD chickens, which was also immunoreactive with the same polyclonal antibody raised against chicken GHBP.

In tissues, the antibody raised against CH17 detected a GHR-related protein of 64 kDa, in addition to the 55 kDa

protein also detected by the GHR/GHBP antibody. The CH17 protein was synthesized from a cDNA clone isolated from an avian hepatic GHR cDNA library screened with a GHR cDNA probe (Bingham *et al.* 1994). The CH17 transcript contains all the extracellular and transmembrane sequences of the GHR and at least 600 bp of the intracellular sequence; however, the use of an alternative splice site results in an insertion and frameshift at position 531 (Bingham *et al.* 1994). The resulting protein would putatively correspond to a membrane-bound GHR variant, deviating from the GHR at amino acid 178 with six novel amino acids and a premature C-terminus. It is therefore pertinent that αCH17 did not detect any immunoreactive proteins in serum. Although the functional significance of the CH17 protein is not known, its ability to recognize a 64 kDa protein in avian liver suggests that it is produced *in vivo*. This truncated membrane-bound protein may be functionally similar to the GHR-like protein recently identified in monkeys (Martini *et al.* 1997) and humans (Ross *et al.* 1997), which negatively inhibits GH action and generates large amounts of binding protein. Additional expression studies would, however, be required to investigate this possibility.

These results indicate that the SLD GHR gene is translated into immunoreactive GHRs and serum GHBPs. As the GHBP is thought to be derived from the extracellular domain of the GHR, a deficiency in membrane GH-binding sites, as demonstrated in the SLD birds, might be expected to be accompanied by a deficiency in serum GH-binding sites. Surprisingly, there was a qualitative difference between serum and hepatic binding sites. Serum GH-binding sites were readily detected (albeit at low levels) in the serum of all SLD birds, whereas hepatic GH-binding activity was undetectable in most birds, although it was detectable in two. Low levels of liver GH binding were also detected in a small proportion of livers from Hubbard strain SLD chickens (Leung *et al.* 1987a), although Leung *et al.* (1987a) did not detect any hepatic GH-binding in the Cornell SLD birds.

Qualitative differences between membrane and serum GH-binding activity are, nevertheless, well documented, as hepatic GHR and serum GHBP levels do not always correlate in chickens (Mao *et al.* 1998), goats (Jammes *et al.* 1996) or pigs (Ambler *et al.* 1992). GH resistance is also associated with detectable serum GHBP in some human populations. Serum GH-binding activity is, for instance, readily detectable in GH-resistant populations of African pygmies (Baumann *et al.* 1989) and the Mountain Ok people of Papua New Guinea (Baumann *et al.* 1991). In LS patients, GHBP activity similarly ranges between normal and undetectable levels (Silbergeld *et al.* 1993, Postel-Vinay 1994, Parks *et al.* 1997). However, in LS, GHBP-positive GH resistance is generally thought to indicate normal hepatic GH-binding sites and downstream defects, such as receptor dimerization or signal transduction (Silbergeld *et al.* 1993, Rosenfeld *et al.* 1994,

Amselem *et al.* 1996). These studies suggest that the deficient GH-binding activity in the SLD hepatic membranes could reflect defective receptor trafficking and/or dimerization rather than, or in addition to, impaired GH affinity.

The quantitative discrepancy between membrane and serum GH-binding activity in SLD birds may have important implications with regard to the origin of avian GHBP. A difference in GHBP and GHR function could putatively result from alternative splicing of GHR gene products producing two distinct mRNAs, as has been shown in rats (Sadeghi *et al.* 1990), mice (Smith *et al.* 1989) and perhaps guinea pigs (Hull *et al.* 1996b). Although multiple GHR-like transcripts are present in chicken liver, exhaustive studies have failed to isolate a transcript that could encode a soluble functional GHBP (Cogburn *et al.* 1997). It is nevertheless possible that a separate transcript that is of low abundance and/or is rapidly degraded encodes an avian GHBP. Indeed, although alternative transcripts of the GHR gene that code for a GHBP were thought to be absent from primates (Leung *et al.* 1987b), a monkey GHBP transcript has recently been cloned that encodes a truncated GHBP (Martini *et al.* 1997).

The avian GHBP may alternatively be derived from proteolytic cleavage of tissue GHRs, as in humans (Leung *et al.* 1987b), rabbits (Leung *et al.* 1987b) and pigs (Bingham *et al.* 1994). If this hypothesis is correct, any defect in the GH-binding domain would abolish binding in the GHR and GHBP to a similar extent. This would suggest that the defect in Cornell SLD birds affects parameters other than, or in addition to, GH-binding ability. It is possible that the GHR gene is translated into functional receptors in Cornell SLD chickens but these proteins may not be correctly inserted into the plasma membrane. Cleavage of the extracellular domain of the receptor into a GHBP could occur intracellularly, as previously shown in rabbits by Amit *et al.* (1993), and GH-binding GHBPs could thus be released into the circulation. If the SLD mutation affects only receptor trafficking, the reduced serum GH-binding activity in SLD chickens would suggest that receptor targeting plays an important role in GHBP synthesis. Alternatively, the mutation in the SLD might impair the stability of the GHR protein to a greater extent than the GHBP. Although Western blot analysis did not reveal any significant variation in steady-state protein levels, a more quantitative study might reveal that SLDs are relatively deficient in the GHR protein.

It is also possible that the SLD serum contains large amounts of a GHBP that is not homologous to the GHR, and that the GHR-like proteins detected by Western blot analysis are dysfunctional. The low-affinity GHBP in humans, for instance, is not immunologically related to the GHR and has recently been shown to be identical with transformed α_2 -macroglobulin (Kratzsch *et al.* 1995).

However, as the SLD GHBP was precipitable by mAb 263, it is likely to be immunologically related to the tissue GHR.

In summary, these results suggest that a missense mutation in the GHR of Cornell SLD chickens may impair the binding ability of the membrane-bound GHR to a greater extent than the serum GHBP.

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