Conversion of Bovine Growth Hormone Cysteine Residues to Serine Affects Secretion by Cultured Cells and Growth Rates in Transgenic Mice

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GHs have been found to possess two disulfide bonds. We set out to determine the importance of bovine (b) GH's disulfide bonds relative to the ability of the hormone to be secreted by cultured cells *in vitro* and to promote growth in transgenic mice. We have generated six mutated bGH genes that encode serine (Ser) substitutions for cysteines (Cys). These mutated genes were used to generate bGH analogs in which either one or both disulfide bonds are destroyed. When the small loop of bGH was destroyed (Cys^{181}-Ser or Cys^{189}-Ser), the bGH analogs were found to be secreted by mouse L-cells at levels comparable to those of wild-type bGH. However, secretion was drastically reduced when the large loop was abolished (Cys^{53}-Ser or Cys^{164}-Ser). An immunofluorescence study of these bGH analogs revealed two distinct patterns of subcellular localization. Bovine GH analogs with mutations in the small loop demonstrated a perinuclear distribution similar to that of wild-type bGH, but analogs containing a disrupted large loop revealed a uniform cytoplasmic distribution pattern. When these mutated bGH genes were individually introduced into transgenic mice, only those animals that expressed bGH analogs with the large loop intact demonstrated a growth-enhanced phenotype. Transgenic mice that expressed bGH analogs lacking the large loop showed growth rates similar to those of nontransgenic mice. These results suggest that the integrity of the large loop, but not that of the small loop, is essential for the growth-enhancing activity of bGH in transgenic mice. (Molecular Endocrinology 5: 598–606, 1992)

INTRODUCTION

GHs are secretory multifunctional polypeptides composed of 191 amino acid residues. They belong to a gene family that also includes PRLs and placental lactogens (1–3). Bovine (b) GH has four cysteine (Cys) residues located at positions 53, 164, 181, and 189, respectively. When aligned to optimize homology, the four Cys residues were found to be invariably conserved among all GHs, PRLs, and PLs (1, 2). This conservation may be indicative of the importance of these Cys residues in a structural and/or functional role. The four Cys residues form two disulfide bridges within the proteins. The bonds are located between Cys^{53} and Cys^{164} (Cys^{53}/Cys^{164}), which results in a large loop, and between Cys^{181} and Cys^{189} (Cys^{181}/Cys^{189}), which results in a small C-terminal loop. Studies have shown that the integrity of the small loop is not essential for the biological activity of bGH (4) or ovine (o) PRL (5).

It has been shown that under identical chemical-reducing conditions, human (h) GH could be completely reduced; however, only the C181/C189 disulfide bond of bGH could be reduced (4). This result suggested that the C181/C189 disulfide bond of bGH is more exposed to reducing agents than the C53/C164 disulfide bond, and that there is also a major difference between bGH and hGH with regard to their ability to form disulfide bonds. When both disulfide bonds of hGH were ruptured and the sulfur atoms carbamidomethylated, full hGH potency was retained in the rat tibia and pigeon crop-sac GH-dependent bioassays (6). In contrast to carbamidomethylated hGH, substantial loss of activity occurred when hGH was reduced and alkylated (7). Loss of bioactivity also was observed when both disulfide bonds forming the large and small loops of porcine (p) GH were reduced and aminoethylated (8). These studies suggest the differential importance of the integrity of disulfide bonds in different species of GHs.

Cysteine residue conversion experiments have been performed for hGH. The Cys^{165} (equivalent to Cys^{164} of bGH) of hGH had been changed to alanine. This hGH analog retained similar immunological activity and possessed full biological activity with respect to wild-type

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hGH (9). The same study also showed that C165A-substituted hGH possessed a mobility similar to that of wild-type hGH when assayed by reducing and denaturing gel electrophoresis; however, a reduced mobility was observed under nonreducing conditions. These results indicate that disruption of the large loop resulted in a significant change in the gel mobility, presumably due to the conformational change in the hGH analog (9). Similar mobility changes were observed for PRLs (10, 11). Together, the above results document the importance of the disulfide bond(s) with respect to the biological activities of GH.

There have not been reports on the effects of alteration of GH disulfide bonds relative to the effects on secretion by *in vitro* cultured cells and on growth of animals. In our previous bGH structure/function studies, secretion alterations in transiently transfected cultured rodent cells were observed for several bGH analogs, including those with alterations in the third α-helix (12) or truncations from the C-terminus (13). In this study, using a site-directed mutagenesis approach, we have systematically substituted Cys^{53}, Cys^{164}, Cys^{181}, and Cys^{189} of bGH to Ser. Cys-Ser conversions were selected to minimize the structural disturbances introduced into the bGH analogs, because Ser has a side-chain similar in size to that of Cys. Mutated bGH genes were subsequently transiently transfected into mouse L-cells to determine whether secretion of the bGH analogs from mouse L-cells were affected by these mutations and to generate sufficient quantities of bGH analogs for GH receptor binding studies. All of the mutated bGH genes were introduced into mouse germ lines to determine whether the growth-promoting activity of bGH was affected in transgenic mice. We have found that disruption of the small disulfide loop does not significantly affect the ability of these GH analogs to be secreted by cultured mouse L-cells, to bind to GH receptors, or to enhance growth in transgenic mice. However, disruption of the large loop drastically affects the intracellular distribution of GH analogs expressed by cultured cells as well as the ability to enhance growth of transgenic mice.

RESULTS AND DISCUSSION

Bovine GH Secretion by Mouse L-Cells

Western Blot Analysis under Reducing Conditions To determine whether the ability to direct GH expression by the altered bGH gene was affected by the mutation, the plasmids were transiently transfected in mouse L-cells. Secretion by L-cells of several of the bGH analogs was found to be drastically affected. All bGH analogs generated in this study demonstrated a mobility identical to wild-type bGH when assayed by reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; Fig. 1-I). Thus, the apparent molecular masses of the analogs are similar (~22 kilodaltons). Two bGH analogs with a single Cys to Ser mutation in the small loop, C181S and C189S, were expressed and secreted at levels comparable to those of wild-type bGH (Fig. 1-I). Apparently, the loss of the small loop had only a minimal effect on the secretion of these analogs. However, secretion of all other bGH analogs, with either single amino acid substitutions in the large loop or double mutations in both the large and small loops, was drastically reduced (Fig. 1-I). This result suggests that an intact large loop structure of bGH is essential for the normal secretion of the protein by cultured L-cells.

Western Blot Analysis under Nonreducing Conditions When analyzed by SDS-PAGE under nonreducing conditions (Fig. 1-II), migration patterns for bGH analogs were more complex. For native bGH, the major extracellular band migrates at the fastest rate relative to other bGH analogs. All other bGH analogs migrate slower than wild-type bGH, apparently due to the fact that they have lost at least one disulfide bond.

Intracellular and extracellular forms of bGH analogs C181S and C189S migrate at a moderately retarded rate compared to native bGH (Fig. 1-II). In contrast to C181S and C189S, bGH analogs C53S, C164S, and C53:C189S, all of which lack the large loop structure, demonstrated drastically reduced migration rates under nonreducing conditions (Fig. 1-II). This result is consistent with that observed for C165A conversion in hGH (9) and for incorrectly disulfide-bonded PRL (10, 11). The loss of one Cys residue involved in formation of the large loop apparently accounts for the large decreases in both the migration rates and secretion levels observed for these two bGH analogs. The large loop structure may play a much more important role than the small loop in maintaining correct overall three-dimensional structure of bGH molecule. The loss of growth-promoting activity by these two bGH analogs in transgenic mice also suggests that C53S and C164S may possess abnormal conformations, which result in a concomitant loss in the ability to bind to GH receptors.

The mobility of the major band for C164S:C181S is surprisingly fast, similar to those of C181S and C189S (Fig. 1-II). Since two Cys residues that are involved in both large and small loop formation were altered, one would expect the migration rate for C164S:C181S to be similar to those of C53S and C164S. However, the result suggests that although C164S:C181S possessed an apparent migration rate similar to those of C181S and C189S, the actual conformation of C164S:C181S might be different from that of C181S and C189S. This may be due to the inability of C164S:C181S to form a normal large loop. A mobility similar to that of C181S and C189S, but faster than that of reduced wild-type bGH (data not shown) suggests that a different type of secondary structure for C164S:C181S does exist under nonreducing conditions. It is possible that instead of the native C53/C164 and C181/C189 disulfide bond formation, the remaining C53 forms a disulfide bond with C189, the only remaining Cys residue in the molecule. This incorrectly disul-

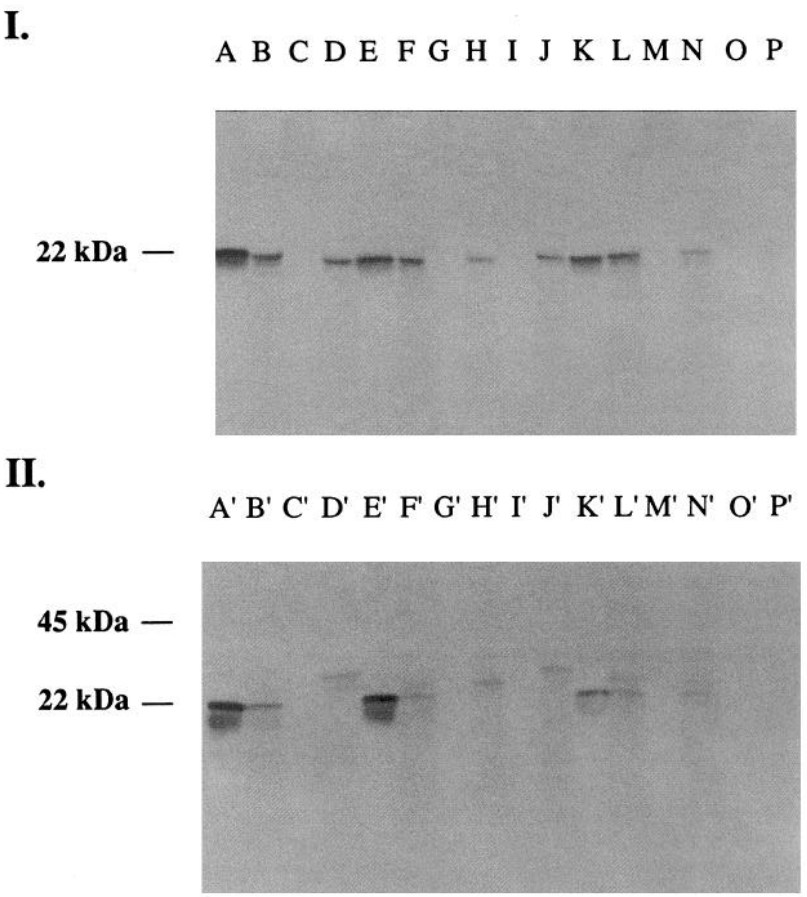

Fig. 1. Western Blot Analyses of bGH and bGH Analogs Derived from Mouse L-Cell Culture Fluids and Cell Lysates

I, Western blot analysis of the bGH and bGH analogs resolved by SDS-PAGE under reducing conditions. Seventy microliters of either cell culture fluids or cell lysates from mouse L-cells transiently transfected with each mutated bGH plasmid DNA were mixed with 7 μl loading dye containing 15% β-mercaptoethanol (2 M, final concentration). After heat denaturation, the samples were subjected to SDS-PAGE (15%). Resolved proteins were transferred to a nitrocellulose membrane. Wild-type bGH and bGH analogs were visualized by a procedure described in *Materials and Methods*. II, Western blot analysis of the bGH and bGH analogs separated by SDS-PAGE under nonreducing conditions. All of the steps were identical to those described above in I, except that the samples were mixed with the loading dye minus β-mercaptoethanol. Lane A, C, E, G, I, K, M, and O in I and II represent culture fluids for native bGH, C53S, C189S, C53S:C189S, C164S, C181S, C164S:C181S, and mock-transfected samples, respectively. Lane B, D, F, H, J, L, N, and P in I and II represent cell lysate samples for native bGH, C53S, C189S, C53S:C189S, C164S, C181S, C164S:181S, and mock-transfected cells, respectively.

fide-bonded bGH analog may assume a relatively compact configuration, which allows faster migration.

Immunofluorescence Study

The subcellular localization of bGH analogs C181S and C189S in L-cells is very similar to that of wild-type bGH (Fig. 2, A, C, and F), in that they display a perinuclear distribution, which we have shown previously (12, 13). Native bGH and these analogs also exhibit a granular distribution pattern. This distribution pattern indicates a normal organized secretory pathway originating from the endoplasmic reticulum and ending in the Golgi apparatus. In constrast, bGH analogs C53S, C53S:C189S, C164S, and C164S:C181S (these alterations resulted in reduced secretion) exhibited a more uniform intracellular distribution pattern, with less granular forms (Fig. 2, B, D, E, and G). This type of distribution pattern is consistent with the earlier observations from our laboratory with other low-secretory bGH analogs (12, 13). A more randomly distributed intracellular bGH suggests that a degradative pathway may be continuously involved in disposing of the incorrectly disulfide-bonded protein. The results of this immunofluorescence study were consistent with findings that proper disulfide bond formation plays an important role in posttranslational and protein-transporting processes (14–16). Recently, immunoglobulin heavy chain binding protein (Bip), found in the endoplasmic reticulum, has been shown to be involved in *in vitro* binding to incorrectly disulfide-bonded polypeptides, including a GH-related, disulfide bond-deficient PRL analog (11). Al-

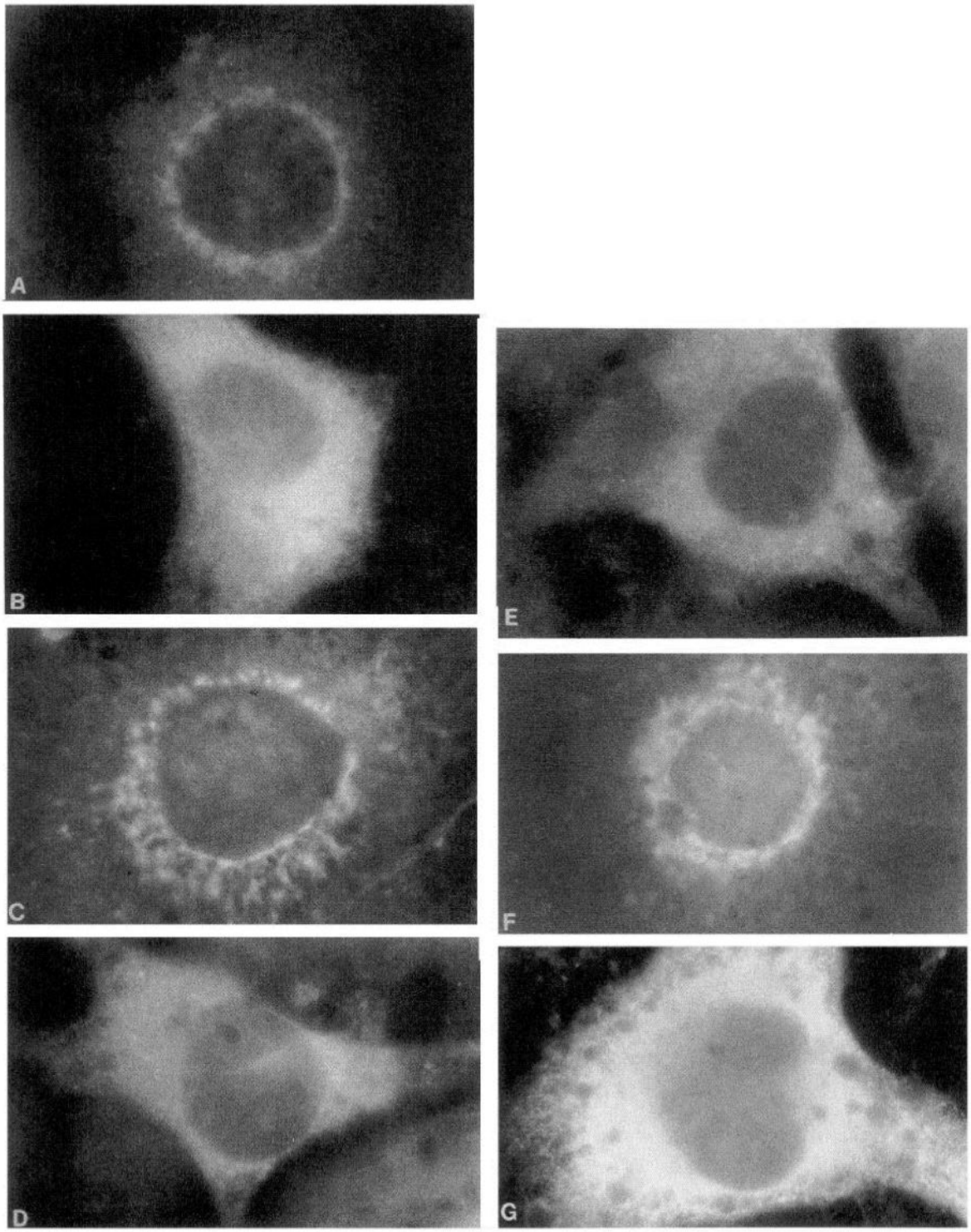

Fig. 2. Fluorescence Microscopy of Mouse L-Cells Transfected with Wild-Type or Mutated bGH Genes Demonstrating Differential Subcellular bGH or bGH Analog Localization

A-G, Mouse L-cells expressing wild-type bGH, C53S, C189S, C53S:C189S, C164S, C181S, and C164S:C181S, respectively. Magnification, ×1000.

though the fate of Bip-bound bGH analogs is not presently known, it is believed that incorrectly disulfide-bonded, Bip-bound polypeptides enter a degradative pathway *in vivo*. Our observation of no obvious increase in intracellular bGH for these low secretory bGH mutations also supports this theory. It would be interesting to determine whether Bip mRNA and protein levels are increased in those mouse L-cells that express low secretory bGH mutations.

Receptor Binding Assay

The receptor binding dissociation constants (K_d) for wild-type bGH, C181S, and C189S, as derived from Scatchard analysis (Fig. 3) were 2.3, 3.0, and 7.4 nM, respectively. The SEMs of all three K_d determinations were no more than 25% of the K_d values. This result suggests that mutation C189S has a minimal effect on the receptor-binding affinity, whereas mutation C181S does reduce, although only moderately (3.2-fold), receptor-binding affinity. The latter result was consistent with that for hGH mutation, in which C182A resulted in a 5.6-fold reduction in the receptor-binding affinity (17). The difference in the reduction may reflect the difference in the two GHs, the difference between the two different mutations, or both.

Transgenic Mouse Study

Transgenic mouse lines were established that express the six different bGH analogs. Numbers of founder mice and transgenic mouse lines established from the founder mice, numbers of transgenes integrated into the mouse genome, as well as ranges of serum concentrations of bGH or bGH analogs found in different founder mice are summarized in Table 1. Western blot analyses of the sera from these transgenic mice revealed that, in contrast to mouse L-cell secretion studies (Fig. 1), the six bGH analogs could be found (three of which are shown in Fig. 4) in the sera of the transgenic mice at levels comparable to those in transgenic mice that express wild-type bGH. This result indicates a major difference between mouse L-cells and transgenic mice in their secretion of incorrectly disulfide-bonded bGH analogs. This result also suggests that the secretion pattern observed in mouse L-cells may be a cell type-specific phenomenon. It is unlikely that the low levels of bGH analogs outside of the L-cells were largely due to the rapid degradation of the proteins, because the same proteins could be found in levels comparable to those of wild-type bGH in the sera of the transgenic mice. Different electrophoretic mobilities demonstrated by serum bGH analogs under nonreducing conditions

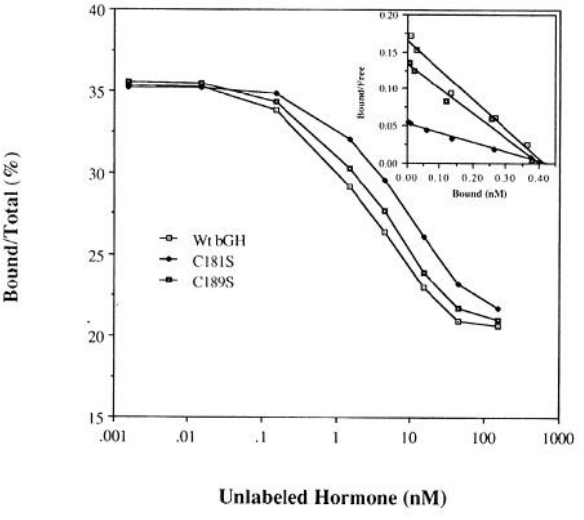

Fig. 3. Displacement Curves for Wild-Type bGH, C181S, and C189S

Receptor binding assays were performed and displacement curves were obtained for wild-type bGH, C181S, and C189S using [^{125}I]hGH and mouse liver membrane preparations. The respective Scatchard plots are shown in the *inset*. Each *data point* represents a mean value calculated from six measurements in two independent receptor binding assays.

Table 1. Summary of Transgenic Mice Expressing bGH or bGH Analogs

Transgene	Transgenic Mice		Transgene Copy No.	Serum bGH (μg/ml)
	Founders	Line(s)		
bGH	7	2	1–20	0.5–5
C53S	4	1	3–10	0.5–3
C189S	3	2	1–20	0.5–3
C53S:C189S	3	1	2–10	1–2
C164S	8	2	2–20	1–5
C181S	4	1	1–20	1–3
C164S:C181S	6	2	2–10	0.5–5

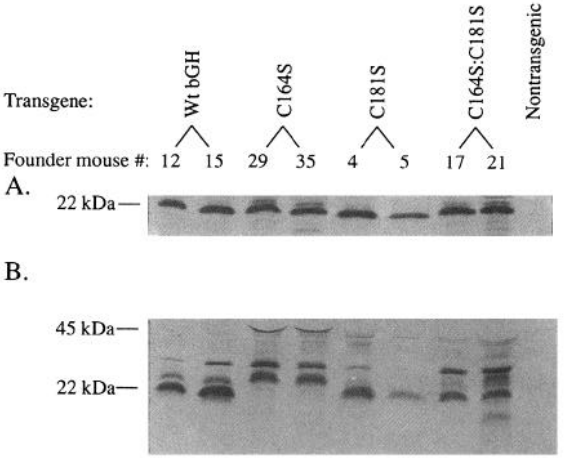

Fig. 4. Western Blot Analysis of Sera from Transgenic and Nontransgenic Mice

Three microliters of serum samples from transgenic mice and nontransgenic mice were subjected to 15% SDS-PAGE under reducing or nonreducing conditions. The remainder of the procedure was identical to that used for analyses of culture fluid and cell lysate samples described in Fig. 1. A, SDS-PAGE under reducing conditions; B, SDS-PAGE under nonreducing conditions.

(Fig. 4B) suggest that Cys-Ser conversions in the analogs persisted in transgenic animals.

Figure 5 demonstrates weight comparisons between male founder transgenic mice that express bGH analogs and animals that express wild-type bGH. Although only male founders were compared in this study, qualitatively similar results were observed when female founders or F1 transgenic mice were used (data not shown). It is interesting to find that the bGH analogs lacking the Cys^{53}/Cys^{164} disulfide bond did not enhance the growth of transgenic mice despite elevated serum levels of bGH analogs. The growth rates of transgenic mice that express bGH analogs C53S, C53S:C189S, C164S, and C164S:C181S are virtually identical to those of nontransgenic mice (Fig. 5). These results were different from previous growth studies on reduced and carbamidomethylated hGH (6) but were consistent with those on reduced aminoethylated pGH (8). Only those bGH analogs that have the Cys^{53}/Cys^{164} disulfide bond intact (C181S and C189S) could enhance growth in transgenic mice ($P < 0.01$ compared with nontransgenic littermates). Although the reasons for this discrepancy between bGH and hGH are not fully understood, one can speculate that chemically modified GHs might be further modified in the bioassay systems used in the previous studies. However, in the transgenic animal system, since Cys residues in the expressed bGH analogs were genetically altered, it is highly unlikely for the genes encoding the substituted Ser residues to back-mutate. The behaviors of these bGH analogs should more accurately reflect the intrinsic biological activities of the mutated bGH. Another possibility is that an incorrect disulfide bond(s) was formed by these bGH analogs. We are currently unable to directly identify an incorrectly formed disulfide bond(s). Both reasonings, nevertheless, suggest that correct folding and the presence of the large disulfide loop are necessary for bGH to exhibit growth-enhancing activity.

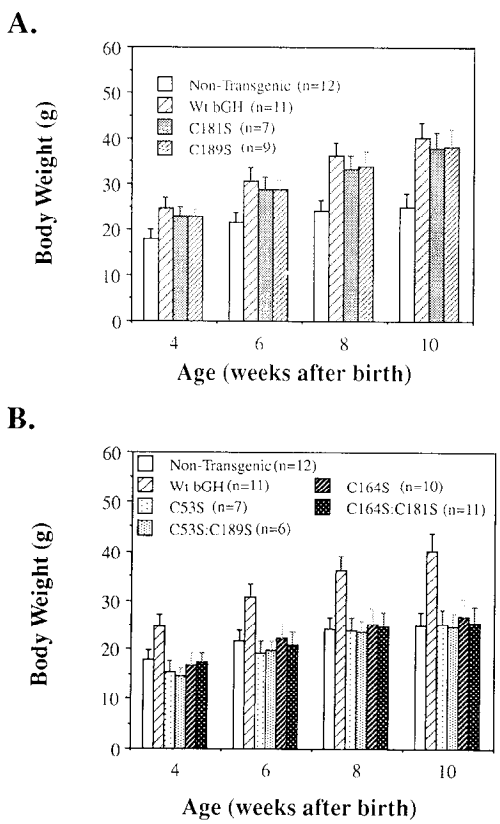

Fig. 5. Comparisons of Growth Rates between Different Lines of Transgenic Mice That Express bGH or bGH Analogs

Weights of transgenic male founder mice expressing bGH or bGH analogs were compared at 4, 6, 8, and 10 weeks of age. The weight is expressed in grams. The n in *parentheses* represents the number of mice analyzed for the comparison. The SDS of the weights are indicated. A shows the growth rate comparison among transgenic mice that express a bGH analog lacking the small loop, transgenic mice that express wild-type bGH, and their nontransgenic littermates. B reveals the growth rate comparison among transgenic mice that express bGH analogs which lack the large loop or the large and small loops, transgenic mice that express wild-type bGH, and their nontransgenic littermates.

Concluding Remarks

Our studies demonstrated that the disulfide bond responsible for the large loop is important for subcellular distribution and secretion of bGH analogs by cultured L-cells, and is also important in enhancing the growth of transgenic mice. This result differs from those found in previous studies using chemically modified hGH (6), but is consistent with those found in the study involving chemically modified pGH (8). The reason for this inconsistency is not fully understood. One explanation is that bGH is different from hGH in the requirement of large loop for growth-promoting activity. However, the possibility that the loss of biological activity by some of the bGH analogs due to the formation of abnormal intramolecular and/or intermolecular disulfide bond(s) during the posttranslational modification process cannot be excluded.

Bovine GH analogs that lack the small loop (C181/C189) were found to be secreted by L-cells, bind GH receptors, and enhance growth in transgenic mice. These results suggest that the small loop is not important in these processes. However, since this small loop is highly conserved among GHs and PRLs, this structure may be important for some general functions that are still unknown.

Our immunofluorescence studies demonstrated two distinctive patterns of subcellular distribution of these bGH analogs in mouse L-cells, which suggests that bGH analogs that lack the large loop may undergo an intracellular trafficking process different from that for native bGH. Also, these bGH analogs that lack the large loop do not enhance growth in transgenic mice. Thus, the integrity of the Cys^{53}/Cys^{164} disulfide bond in bGH is essential for growth enhancement in the GH transgenic mouse model.

MATERIALS AND METHODS

Materials

Iodinated hGH ([^{125}I]hGH; ~100 μCi/ml) was obtained from New England Nuclear (Boston, MA). Rabbit anti-bGH antibodies were a gift from Dr. Fritz Rottman, Case Western Reserve University. Dulbecco's Modified Eagle's Medium (DMEM) was obtained from Sigma Chemical Co. (St. Louis, MO).

Generation of bGH Mutations

The pbGH10Δ and pbGH10Δ16 (Fig. 6, A and B) were used as parental plasmids for site-directed mutagenesis. Each plasmid contains a 1.7-kilobase (kb) metallothionein transcriptional regulatory sequence linked to a bGH DNA sequence. The differences between two plasmids are that pbGH10Δ contains 2.4 kb genomic bGH sequence, while pbGH10Δ16 contains the same bGH sequence with second and third introns (introns b and c) removed. Each of these fusion genes was expressed in cultured mouse L-cells and transgenic mice (data not shown). Unique restriction enzyme cleavage sites, *Acc*I (Fig. 6A), *Mlu*I, and *Pvu*II (Fig. 6B), were introduced into the plasmids to facilitate generation of mutations. The pbGH10Δ was used to generate the mutations C53S, C189S, and C53S:C189S using a method involving linearized and gapped-linearized DNA segments (18). Mutations C164S, C181S, and C164S:C181S were generated from pbGH10Δ16 using site-directed oligonucleotide-specific mutagenesis, as described previously (19). Oligonucleotides were generated using a DuPont Coder 300 DNA synthesizer (Wilmington, DE). Mutations within the bGH gene were confirmed by the dideoxy chain termination method (20), using modified T7 DNA polymerase (Sequenase, U.S. Biochemical Corp., Cleveland, OH) and purified oligonucleotides as primers (21).

Mouse L-Cell Culture and Transient Transfection

Mouse L-cells were maintained in six-well cell culture plates in DMEM supplemented with 10% NuSerum (Collaborative Research, Waltham, MA), as previously described (22). Four micrograms of plasmid DNA in 1 ml DMEM (minus serum) were used to transiently transfect approximately 1×10^6 cells using the DEAE-dextran-dimethylsulfoxide shock protocol (12). Medium was changed every 24 h. Culture fluids 5 days posttransfection were collected and analyzed as discussed below. The cells in each well were washed with PBS and lysed by the addition of 0.5 ml 0.1% Triton X-100 in 100 mM Tris (pH 7.8). Intracellular proteins were separated from cell debris by centrifugation.

SDS-PAGE and Western Blot Analysis

Protein samples found in the culture fluid or cell lysates were subjected to 15% SDS-PAGE. Seventy microliters of sample were loaded in each well. SDS-PAGE was performed under reducing and nonreducing conditions. Protein samples were mixed with loading dye containing 15% β-mercaptoethanol for reducing gels and without β-mercaptoethanol for nonreducing gels. Other conditions for reducing and nonreducing gels were identical. After electrophoresis, proteins were transferred to nitrocellulous membranes (23). Bovine GH or bGH analogs were identified by first incubating the membrane with rabbit anti-bGH antibodies and subsequent incubating them with goat antirabbit immunoglobulin G antibody conjugated with horseradish peroxidase (12). Western analysis of serum samples from transgenic mice was performed similarly, except only 3 μl serum were used for each sample.

Immunofluorescence Study

Subcellular localization of bGH was performed using a modification of a previously described procedure (12, 24). Mouse

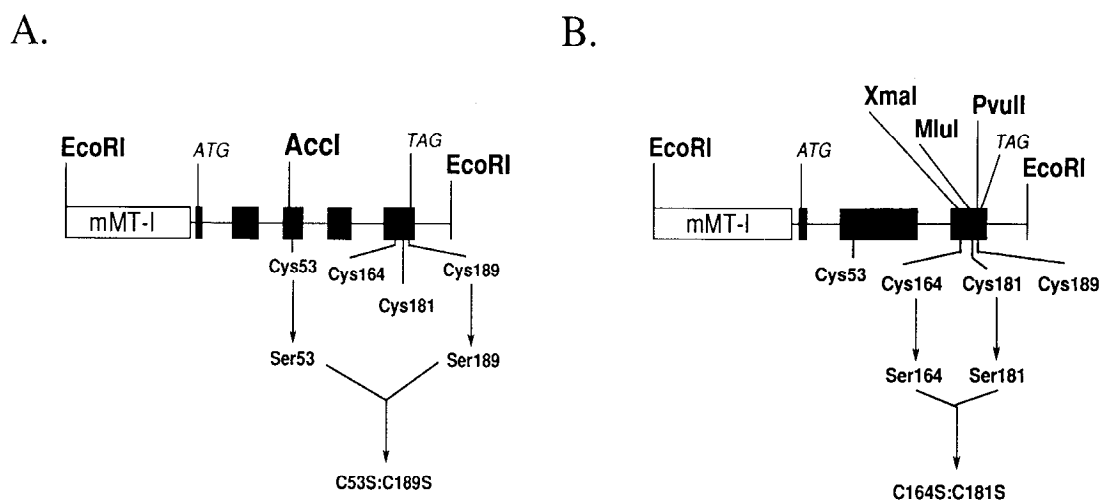

Fig. 6. Schemes for Generating Site-Directed bGH Mutations

A, Plasmid pbGH10Δ was used for generating C53S, C189S, and C53S:C189S mutations. It contains a 2.3-kb bGH genomic DNA sequence [five exons (*black boxes*) and four intervening sequences] linked to a 1.7-kb mouse metallothionein-I transcriptional regulatory sequence. A unique restriction site, *Acc*I, located near the codon for Cys^{53} was inserted to facilitate plasmid screening. B, The pbGH10Δ16 possesses the same DNA composition as pbGH10Δ, except that introns b and c have been removed. In addition, two unique restriction sites, *Mlu*I and *Pvu*II, were inserted to facilitate oligonucleotide-directed mutagenesis. The locations of the four cysteine residues that form the two disulfide bonds in bGH are shown in both A and B. Each mutation generated from the parental plasmids is indicated. Double mutations, C53S:C189S and C164S:C181S, were made by ligation reactions of DNA fragments containing the single mutations. Transgenic mice were generated by microinjection of *Eco*RI/*Eco*RI DNA fragments of 4.0 kb (A) or 3.6 kb (B).

L-cells grown on glass coverslips were transiently transfected with wild-type or mutant bGH plasmids. Five days posttransfection, the cells were fixed in a 3% paraformaldehyde-PBS solution for 30 min and then permeabilized for 15 min in 0.1% Nonidet P-40 and 1% BSA in PBS at room temperature. The cells were rinsed twice with 1% BSA in PBS (5 min/wash) and blocked with normal goat serum (1:100 diluted in 1% BSA-PBS) for 1 h at room temperature. After rinsing three times with 1% BSA in PBS, the cells were incubated overnight with rabbit anti-bGH serum, rinsed three times with 0.5% Tween-20 and 1% BSA in Tris-buffered saline (TBS), followed by 1-h incubation at room temperature with a goat antirabbit biotin conjugate (1:100 diluted in 0.5% Tween-20 and 1% BSA in TBS). The coverslips were rinsed with 0.5% Tween-20 and 1% BSA in TBS and then incubated for 30 min with streptavidin-fluorescein isothiocyanate. Specimens were mounted for immunofluorescence microscopy.

Receptor Binding Experiment

Bovine GH and bGH analogs, C181S and C189S, found in the cell culture fluid were individually concentrated using an ultrafiltration method (Amicon, Danvers, MA). Other mutations could not be isolated at sufficient quantities for receptor binding studies due to their very low secretion levels. The concentration of each protein was determined by Western blot analyses (25). Male mouse liver membrane preparations were made, and RRAs were performed as previously described (12). Bovine GH and bGH analogs were used to compete with [^{125}I]hGH for binding to mouse GH receptors. A Scatchard analysis function of a computer program, Ligand (26), was used to determine receptor-binding affinities for bGH and bGH analogs based upon assumptions that bGH and bGH analogs are homologous to hGH and can be treated as the same molecule.

Trangenic Mice and Statistical Analysis

Approximately 2–4 pg Met-bGH or plasmids encoding the Cys to Ser mutations were cleaved with *Eco*RI. A ~3.0-kb DNA fragment was used for microinjection into fertilized mouse embryos, using a standard protocol, described previously (27). One month after the mice were born, transgenic mice were identified by DNA slot blot analysis of the mouse tail DNA, using ^{32}P-labeled bGH as probe (data not shown). Expression of the transgenes was demonstrated by Western blot analysis of serum proteins from those transgenic mice. The growth rates of different transgenic mouse lines and nontransgenic mice were monitored at biweekly intervals from 4–10 weeks of age. Statistical analyses were performed using an ungrouped Student's t test on the growth rates of different transgenic mouse lines. The significance level was set at $P < 0.01$.

Acknowledgments

The authors thank Paul Wiehl for generating oligonucleotides, Eric Holley for technical assistance with trangenic mice, and Bruce Kelder for helpful comments.

Received September 4, 1991. Rerevision received February 4, 1992. Accepted February 4, 1992.

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* Supported by the state of Ohio's Eminent Scholar Program, which includes a grant from Milton and Lawrence Goll.

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Announcement from the American Board of Internal Medicine: Addition of Diabetes to the Name of the ABIM Subspecialty Board on Endocrinology and Metabolism

In September 1991, the American Board of Medical Specialties approved the request of the American Board of Internal Medicine to change the name of its Subspecialty Board on Endocrinology and Metabolism to the Subspecialty Board on Endocrinology, Diabetes, and Metabolism.

The rational for the change is 2-fold: to recognize the increasing complexity involved in the care of patients with diabetes mellitus and its complications; and to recognize that the care of patients with diabetes is integral to the training and expertise of the certified endocrinologist.

Since certification in endocrinology and metabolism was introduced by the board in 1972, major advances have occurred in the management of diabetic patients. The Special Requirements of the Accreditation Council for Graduate Medical Education specify that the training curriculum in endocrinology and metabolism must provide opportunities for the trainee to develop broad clinical competence in the management of patients with diabetes mellitus, including management of acute and chronic complications. The board had kept pace with these developments in its certification process. Approximately 25% of the current certifying examination is devoted to questions that focus on diabetes mellitus and its complications. The change in the name of the Subspecialty Board provides recognition of the importance of diabetes care to the subspecialty.

Beginning with the 1991 certifying examination, all successful candidates for certification and recertification in the subspecialty will receive certificates bearing this new name. The Board will not provide new certificates to its 2760 diplomates previously certified in endocrilology and metabolism. However, it is understood that certification in endocrinology and metabolism before 1991 also confers recognition of special expertise in diabetes mellitus.