ABSTRACT  The anterior pituitary gland produces a 20-kilodalton (kDa) variant of human growth hormone (hGH) that differs from the predominant 22-kDa form of hGH in that amino acid residues 32–46 are deleted. Previous work has suggested that the 20-kDa variant possesses the full growth-promoting and lactogenic activities of 22-kDa hGH but lacks its intrinsic diabetogenic and insulin-like activities. In the present study, recombinant DNA techniques were used to prepare biosynthetic 20-kDa hGH, and some of the biological properties of the purified hGH variant were examined. The biosynthetic 20-kDa hGH variant was found to share the propensity for agglutination exhibited by its native counterpart. Moreover, like the native variant, biosynthetic 20-kDa hGH possessed full growth-promoting activity in the weight gain test in hypophysectomized rats. However, contrary to previous work suggesting that native 20-kDa hGH lacks diabetogenic and insulin-like activities, biosynthetic 20-kDa hGH was found to have substantial diabetogenic activity when administered chronically to ob/ob mice and to possess approximately 20% of the in vitro insulin-like activity of biosynthetic 22-kDa hGH on isolated epididymal adipose tissue of hypophysectomized rats. The diabetogenic and insulin-like activities of biosynthetic 20-kDa hGH cannot be ascribed to contamination of the hormone preparation with the 22-kDa form of hGH or with other diabetogenic or insulin-like pituitary peptides. Therefore, the results strongly suggest that diabetogenic and insulin-like activities are also intrinsic properties of the 20-kDa variant of hGH.

A 20-kilodalton variant of human growth hormone (20-kDa hGH) has been isolated from human pituitary glands (1) and has been found to differ from the predominant 22-kilodalton form of hGH (22-kDa hGH) in that residues 32–46 are deleted (2, 3). The variant is produced in the human pituitary, because the nucleotide sequence encoding residues 32–46 is sometimes excised during the processing of hGH pre-mRNA to hGH mRNA (4). As a consequence, approximately 5–10% of the hGH present in the human pituitary is 20-kDa hGH (1). Physicochemical studies of 20-kDa hGH (3) suggest that its conformation is similar but not identical to that of 22-kDa hGH.

The 20-kDa variant of hGH has been reported (1, 5) to have growth-promoting activity in the hypophysectomized rat equivalent to that of 22-kDa hGH. Moreover, its lactogenic activity in the pigeon crop sac assay equals that of 22-kDa hGH (1). On the other hand, Lewis et al. (5) reported that 20-kDa hGH lacked diabetogenic activity, in that it failed to produce hyperglycemia and glucose intolerance in dogs, when administered 10 hr prior to a glucose tolerance test. Also, this same group reported (6) that 20-kDa hGH lacked the acute insulin-like property of 22-kDa hGH, since the 20-kDa hGH variant failed to produce hyperglycemia and increase plasma free fatty acids when administered to hypophysectomized rats and did not increase glucose uptake or oxidation to CO₂ by isolated rat adipose tissue when added in vitro. These observations have led to the conclusion that, unlike 22-kDa hGH, the 20-kDa variant lacks significant diabetogenic and insulin-like activities. The explanation proposed is that the active site(s) for these properties of hGH reside in the region of the amino acid deletion or because conformational changes in the molecule resulting from the deletion interfere with the expression of these properties (5, 7).

The gene for 22-kDa hGH has been cloned and expressed in bacteria, and the biosynthetic 22-kDa methionyl-hGH (22-kDa Met-hGH) has been characterized (8). Like its native counterpart, the biosynthetic hormone not only is growth promoting (8, 9) but also has diabetogenic (10–12) and insulin-like activities (13). Since decreased glucose tolerance and increased insulin resistance are observed upon treatment with both 22-kDa hGH and 22-kDa Met-hGH at growth-promoting doses (see, for example, ref. 10), the use of hGH at higher doses for indications other than to promote growth may be limited. If 20-kDa hGH does indeed lack diabetogenic activity it could be preferable to 22-kDa Met-hGH as an anabolic hormone. In the present study, bacterially derived biosynthetic 20-kDa Met-hGH was produced and characterized. A major advantage accruing from the use of bacterially derived biosynthetic 20-kDa Met-hGH is that contamination with the 22-kDa form of hGH is obviated, which in the case of purified native 20-kDa hGH is very difficult to achieve (5).

MATERIALS AND METHODS

Biosynthetic 22-kDa Met-hGH was prepared and characterized as previously described (8). Biosynthetic 20-kDa Met-hGH was prepared as follows. The gene constructed for the production of 22-kDa Met-hGH (14) served as the starting material for the construction of the gene for 20-kDa Met-hGH. A synthetic oligodeoxynucleotide was annealed with the coding strand of 22-kDa hGH DNA, joining the anticodons of amino acids 31 and 47. Primed synthesis of DNA in the presence of T4 DNA ligase resulted in synthesis of a shorter gene incorporating the annealed deoxyoligonucleotide. This in vitro deletion mutagenesis of the 22-kDa hGH gene resulted in the DNA sequence for amino acid residues 32–46 being looped out of the newly synthesized DNA. The resulting gene was then incorporated into the plasmid, which was used to transform Escherichia coli (15). E. coli cells containing the plasmid with the 20-kDa hGH gene were grown to an OD₅₅₀ of 50. The cells were harvested by centrifugation and then frozen. The 20-kDa Met-hGH was extracted from the cells after they were held at −20°C for 48 hr or longer. The 20-kDa Met-hGH was purified to homogeneity from the

Abbreviation: hGH, human growth hormone.
E. coli extract by using standard techniques, including DEAE-cellulose ion-exchange chromatography, gel permeation chromatography, ammonium sulfate precipitation, and ultrafiltration. The purified 20-kDa Met-hGH was dissolved in 90 mM mannitol/5 mM sodium phosphate, pH 7.9, and lyophilized.

The growth-promoting activity of 20-kDa Met-hGH was assessed by using the weight gain test in hypophysectomized rats as previously described (8). Diabetogenic activity was determined by the effect of a 3-day course of hormone treatment on fasting blood glucose levels and glucose tolerance in the hereditarily obese (ob/ob) mouse (C57BL6 ob/ob, The Jackson Laboratory). The methods used have been described in detail (11). In vitro insulin-like activity was assessed by measuring the ability of the hormone preparations to stimulate D-[U-14C]glucose oxidation to 14CO2 by isolated epididymal adipose tissue of hypophysectomized rats (Charles River Breeding Laboratories) weighing approximately 100 g. The details of the procedure have been described (16).

RESULTS

Because of the similarities in size and properties of native 22-kDa hGH and 20-kDa hGH, it has been quite difficult in the past to isolate 20-kDa hGH from pituitary extracts in a form free of contamination with small amounts of 22-kDa hGH and other pituitary hormones. In contrast, in the present study it was possible, by using recombinant DNA techniques, to produce biosynthetic 20-kDa hGH that is not contaminated with these substances. As would be expected from the techniques chosen to construct the gene for 20-kDa hGH, the biosynthetic hormone contains an NH2-terminal methionine, as does biosynthetic 22-kDa hGH (8). That the biosynthetic 20-kDa Met-hGH possesses the correct amino acid sequence was verified by restriction mapping of the constructed 20-kDa hGH gene and by tryptic peptide mapping of the purified 20-kDa Met-hGH. The purity of the 20-kDa Met-hGH preparation was assessed by NaDodSO4 gel electrophoresis and isoelectric focusing. NaDodSO4 gel electrophoresis was performed as described by Laemmli (17), and the gels were silver stained according to the procedure of Merrill et al. (18). Fig. 1 shows the electrophoretic patterns obtained with 20-kDa Met-hGH, 22-kDa Met-hGH, and a sample of pituitary 22-kDa hGH that served as the standard. Electrophoresis was performed in the presence and absence of the reducing agent 2-mercaptoethanol. Protein aggregating through covalent disulfide linkages can be reduced to monomers with 2-mercaptoethanol. The similarity of the biosynthetic 20-kDa and 22-kDa Met-hGH electrophoretic patterns in the presence and absence of 2-mercaptoethanol is indicative of the absence of any significant covalent aggregation. It can be seen that 20-kDa Met-hGH had a mobility greater than that of 22-kDa Met-hGH or native 22-kDa hGH, reflecting the lower molecular weight of the biosynthetic 20-kDa Met-hGH. A trace of native 20-kDa hGH can be seen in the pituitary 22-kDa hGH sample migrating faster than the main band. The absence of other staining bands on the gels of 20-kDa Met-hGH and 22-kDa Met-hGH suggested that these substances are greater than 99% pure, as assessed by this technique.

The 20-kDa Met-hGH preparation was also submitted to isoelectric focusing according to the procedure provided by LKB, using their pH 4.0–6.5 PAG Plate Ampholine-containing gels. The results obtained comparing 20-kDa Met-hGH and 22-kDa Met-hGH are shown in Fig. 2. It can be seen that 20-kDa Met-hGH has a more basic isoelectric point than 22-kDa Met-hGH, as was expected from previous work (1) on native 20-kDa hGH. This results from the absence in 20-kDa hGH of several acidic amino acid residues present in pos-

**FIG. 1.** NaDodSO4 gel electrophoresis of various hGH preparations. Protein bands were visualized by using silver-staining techniques. The relative mobilities of proteins are indicated by various marker proteins of known molecular mass.

**FIG. 2.** Narrow-range isoelectric focusing of Met-hGH preparations. Protein bands were visualized after staining with Coomassie blue. The pI values of various marker proteins are indicated.
mouse. In these experiments, groups of ob/ob mice were injected subcutaneously (s.c.) for 3 consecutive days with saline, and on the fourth day they were given 2 μg of dexamethasone phosphate s.c., fasted for 6 hours, and then given an intraperitoneal glucose tolerance test. Seven days after the start of the saline injections, the same mice were treated for three days with 22-kDa Met-hGH, and on the fourth day the tolerance test was repeated. In each panel in Fig. 3, the saline control curve was obtained from the same animals used for the corresponding hGH treatment. It can be seen in Fig. 3 that a dose of 10 μg/day of 22-kDa Met-hGH produced marginal effects on fasting blood glucose concentration and glucose tolerance. However, doses of 25 or 50 μg/day produced unequivocal increases in fasting blood glucose concentration and impairment of glucose tolerance. Similar experiments employing 20-kDa Met-hGH are illustrated in Fig. 3 Lower. It is clear that 20-kDa Met-hGH produced a marked increase in fasting blood glucose concentration and an impairment in glucose tolerance when administered for 3 days at doses of 25 or 50 μg/day. Essentially identical results were obtained with 20-kDa Met-hGH in another series of experiments (results not shown). Thus, 20-kDa Met-hGH has unequivocal diabetogenic activity when given chronically to ob/ob mice, and the magnitude of its effect is approximately that of 22-kDa Met-hGH.

Biosynthetic 22-kDa Met-hGH and 20-kDa Met-hGH were also tested for the ability to stimulate [14C]glucose oxidation to 14CO2 in vitro by isolated epididymal adipose tissue of hypophysectomized rats. Preliminary experiments indicated a significantly smaller effect of 20-kDa Met-hGH in this system. Consequently, higher concentrations of 20-kDa Met-hGH were used to obtain an estimate of its relative activity. The results obtained are shown in Fig. 4. It can be seen that the in vitro insulin-like activity of 22-kDa Met-hGH appeared to be comparable to that of 5 times the concentration of 20-kDa Met-hGH. Therefore, while 20-kDa Met-hGH clearly retains the insulin-like property of growth hormone, this activity is approximately 20% that of 22-kDa Met-hGH.
DISCUSSION

Bacterially derived biosynthetic 20-kDa Met-hGH was isolated and chemically characterized. The purified hGH variant was found to share the propensity for aggregation exhibited by its native counterpart. Moreover, like the native hGH variant, 20-kDa Met-hGH possessed full growth-promoting activity in the weight gain test in hypophysectomized rats. However, contrary to previous observations (5, 6) suggesting that native 20-kDa hGH lacks diabetogenic and insulin-like activities, biosynthetic 20-kDa Met-hGH was found to have substantial diabetogenic activity when administered chronically to ob/ob mice and to possess approximately 20% the in vitro insulin-like activity of biosynthetic 22-kDa Met-hGH. Since bacterially derived biosynthetic 20-kDa hGH was used, the finding of diabetogenic and insulin-like activities cannot be ascribed to contamination of the hormone preparation with 22-kDa hGH or other diabetogenic or insulin-like pituitary peptides. The extent of aggregation was much smaller than the magnitude of change in the insulin-like activity observed. This, coupled with the fact that the diabetogenic response was nearly the same as to 22-kDa hGH, suggests the small degree of aggregation had little effect on the biological activity. Therefore, the results of the present study strongly challenge the conclusion (5, 6) that the 20-kDa variant of hGH lacks the diabetogenic and insulin-like properties of 22-kDa hGH.

The failure of Lewis and his colleagues to detect diabetogenic (5) or insulin-like (6) activities in native 20-kDa hGH may be related to the nature of the assays used in their studies. To test for the diabetogenic activity of 20-kDa hGH, Lewis et al. (5) used an acute (10-hr) glucose tolerance assay in the intact dog. Unfortunately, 22-kDa hGH itself exhibits little diabetogenic activity under these conditions. At least 2 days of treatment with growth hormone are required (21, 22) to produce hyperglycemia and glucose intolerance in the dog. Thus, it is not surprising that native 20-kDa hGH did not cause hyperglycemia and glucose intolerance in the dog 10 hr after its administration (5). In the experiments in which the insulin-like activity of native 20-kDa hGH was assessed (6), only one dose of 20-kDa hGH was used, and the results obtained were compared to those produced with an equivalent dose of native hGH. It is possible that the insulin-like activity of native 20-kDa hGH was not detected because its insulin-like activity may be significantly attenuated, as is that of biosynthetic 20-kDa hGH, and that the single dose employed was below that required to elicit a response.

Since biosynthetic 20-kDa Met-hGH exhibited the diabetogenic and insulin-like properties typical of native 22-kDa hGH, it seems reasonable to conclude that residues 32–46 of the hGH molecule are not obligatory for the expression of these activities. However, 20-kDa Met-hGH had only 20% the insulin-like activity of 22-kDa Met-hGH, and consequently we infer that the deleted region may be required for the full expression of activity, either because it contains a portion of the active site for this property or because the conformation of the 20-kDa variant interferes with the full expression of insulin-like activity. In this regard, a synthetic peptide corresponding to residues 31–44 of hGH has been reported to stimulate glucose uptake by isolated rat epididymal adipose tissue (23). On the other hand, it is worth noting that even structural modifications of the hGH molecule not involving amino acid deletions (e.g., S-carboxymethylalanine) frequently result in the attenuation of insulin-like activity (24).

The exact nature of the mechanism by which growth hormone is involved in glucose metabolism is unclear. Until the receptors for hGH have been characterized and the subsequent chain of events defined, it will be difficult at best to ascribe various biological activities to different growth hormone forms and fragments. An understanding of the mechanisms at the molecular level will enhance the understanding of various biological activities observed and permit tailor-made forms of growth hormone to target only the actions desired, with the potential of reducing or eliminating biological activities that are deemed undesirable.

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