Recombinant Hormones from Fragments of Human Growth Hormone and Human Placental Lactogen*

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Peptide fragments generated by limited plasmin digestion and reduction of human growth hormone (hGH) and human placental lactogen (hPL) were recombinated to produce biologically active molecules consisting of the NH2-terminal two-thirds of one hormone molecule linked to the COOH-terminal one-third of the other. Plasmin-cleaved hPL and hGH each consist of an NH2-terminal fragment (1-134) connected to a COOH-terminal fragment (141-191) through a Cys88-Cys151 disulfide bond. The plasmin-cleaved hormones were reduced, the fragments isolated, and then recombined in equimolar quantities to prepare recombinant hormones. On sodium dodecyl sulfate gel electrophoresis the recombinants migrated as a single band in the position of native hormone when they were not reduced and as two bands in the positions of the fragments when they were reduced. This indicated that the recombinants had re-formed a disulfide bond. The derivatives were tested for immunologic activity in hPL and hGH radioimmunoassays, for lactogenic and growth-promoting activity by the radioreceptor assays using membranes from mammary gland and liver of lactating rabbits, and for secondary and tertiary structure by circular dichroism measurements in the near and far UV regions. The recombinant with NH2-terminal hGH had hGH immunologic activity, hGH structure and lactogenenic and growth-promoting activity, while the hybrid with NH2-terminal hPL had hPL immunologic activity, hPL structure, and lactogenetic activity alone. Therefore the immunologic activity, biologic activity, and structure of each of the covalently linked derivatives was characteristic of the hormone's NH2-terminal 1-134 fragment. The COOH-terminal 141-191 sequence maintains overall conformation but appears to have little, if any, role in determining biologic specificity.

Characterization of the plasmin fragments of hGH (4-6) and hPL, (7) has provided evidence that biologic activity in each hormone is associated with the NH2-terminal two-thirds of the molecule. However, differences in the amino acid sequence of the COOH-terminal fragments could play a role in the 109-fold difference in growth-potentiating activity which exists between hGH and hPL. In order to determine the role of individual regions of the hormone, hybrids consisting of the NH2-terminal fragment of one hormone covalently linked through the natural disulfide bond to the COOH-terminal fragment of the other hormone were prepared. This paper describes the synthesis and characterization of two covalently linked recombinant hormones: hybrid-hPL (hPL 1-134-S-S-hGH 141-191) and hybrid-hGH (hGH 1-134-S-S-hPL 141-191).

MATERIALS AND METHODS

Preparation of Plasmin-cleaved hPL and hGH—The hPL used in these studies was kindly supplied by Drs. Paul Bell and C. Breuer of Lederle, and was subjected to further purification by gel filtration over a column (5.0 X 95 cm) of Sephadex G-100 eluted with 0.05 M NH4HCO3. HGH, Lot HS 1373C, was obtained from the National Pituitary Agency, National Institute of Arthritis, Metabolism, and Digestive Diseases, National Institutes of Health. Human placasin was generously provided by Dr. Kenneth Robbins and was prepared according to the method of Robbins and Summara (8). HPL was digested with a 1:50 (w/w) ratio of plasmin for 12 h at 37°C. HGH was digested with a ratio of 1:100 (w/w) for 1 h at 37°C. In both cases the protein digestes were applied to columns (5.0 X 95 cm) of Sephadex G-100 and eluted with 0.05 M NH4HCO3. The protein fractions which eluted in the same position as native hPL or hGH were pooled and lyophilized.

Isolation of Reduced and Alkylated Fragments from PL-hGH and PL-hPL—Plasmin fragments were isolated as their S-carbamidomethylated derivatives by methods previously published (3).

Isolation of Plasmin Fragment with Free Sulphydryl Groups—Twenty milligrams of plasmin-cleaved hGH or plasmin-cleaved hPL were dissolved in 5 ml of buffer containing 0.05 M NH4HCO3, and 8 M urea. After dialysis with N4, 50 μl of β-mercaptoethanol were added, and the mixture was held for 2 h at 37°C. Reduced fragments were separated by gel filtration on a column (2.5 X 95 cm) of Sephadex G-100 eluted with 0.05 M NH4HCO3 containing 8 M urea and 6.01% diithiothreitol to prevent formation of disulfide bonds. Following purification, urea and diithiothreitol were removed by gel filtration of each of the peaks on a column (2.5 X 45 cm) of Sephadex G-55 eluted with 0.05 M NH4HCO3.

Recombination of S-Carbamidomethylated Plasmid Fragments—The S-carbamidomethylated fragments were dissolved in 0.05 M NH4HCO3 to give equimolar concentrations. Exact concentrations were determined by the method of Lowry (9) and by amino acid analysis. In a typical recombination experiment, 1.65 mg (100 nmol) of RCAM-hPL-1-134 were added to 0.55 mg (100 nmol) of RCAM-hGH-141-191 and incubated for 10 days (10). The recombinant fragments were passed over α column (1.5 X 95 cm) of Sephadex G-100 eluted with 0.05 M NH4HCO3. The protein which eluted in the position of intact hPL (or hGH) was used in the studies described herein.

Formation of Disulfide-linked Recombinates—The nonalkylated fragments were combined in the proportions described above and...
of hGH and hPL. The analyses were uncorrected for losses due to hydrolysis. Samples of hPL were reduced before boiling.

Based on values of aspartic acid determined from sequence analysis of hGH and hPL, the analyses were uncorrected for losses due to hydrolysis.

TABLE I

Amino acid analyses of the hybrid hormones

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>hPL hybrid</th>
<th>Expected values</th>
<th>hGH hybrid</th>
<th>Expected values</th>
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<tbody>
<tr>
<td>Half-cystine</td>
<td>3.5</td>
<td>4</td>
<td>3.7</td>
<td>4</td>
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<tr>
<td>Aspartic acid</td>
<td>23</td>
<td>23</td>
<td>19</td>
<td>19</td>
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<tr>
<td>Threonine</td>
<td>10.4</td>
<td>11</td>
<td>8.6</td>
<td>9</td>
</tr>
<tr>
<td>Serine</td>
<td>16.7</td>
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<tr>
<td>Glutamic acid</td>
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<td>24</td>
<td>26.4</td>
<td>26</td>
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<tr>
<td>Proline</td>
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<td>5</td>
<td>6.7</td>
<td>8</td>
</tr>
<tr>
<td>Glycine</td>
<td>5.7</td>
<td>6</td>
<td>7.4</td>
<td>7</td>
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<td>Alanine</td>
<td>5.5</td>
<td>6</td>
<td>7.3</td>
<td>7</td>
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<tr>
<td>Valine</td>
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<td>7</td>
<td>6.7</td>
<td>7</td>
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<td>6</td>
<td>3.5</td>
<td>4</td>
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<td>Isoleucine</td>
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<td>7</td>
<td>6.2</td>
<td>6</td>
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<td>24.8</td>
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<td>Tyrosine</td>
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<td>8</td>
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<td>12</td>
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<tr>
<td>Histidine</td>
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<td>6</td>
<td>3.4</td>
<td>4</td>
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<td>Lysine</td>
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<td>8</td>
<td>7.8</td>
<td>8</td>
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<tr>
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<td>1</td>
<td>0.8</td>
<td>1</td>
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<tr>
<td>Arginine</td>
<td>10.3</td>
<td>11</td>
<td>10.4</td>
<td>11</td>
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</tbody>
</table>

* Derived from the amino acid sequence of hPL and hGH fragments 1–134 and 141–191.

** Quantitated as S-carboxymethylcysteine following reduction and alkylation with iodoacetamide.

† Determined by hydrolysis in 40% methane sulfonic acid (12).

Lyophilized. The lyophilized mixture of fragments was redissolved in 1 ml of buffer containing 0.05 M NH₄HCO₃, 8 M urea, and 2% mercaptoethanol. The solution was kept for 2 h at 37°C and applied to a column (1.5 × 45 cm) of Sephadex G-25 eluted with 0.05 M NH₄HCO₃. The recombinants appeared in the void volume free from urea and reducing agent. When SDS-gel electrophoresis indicated the presence of any uncombined fragments, the hybrids were purified further on a column of Sephadex G-100 (1.5 × 95 cm) eluted with 0.05 M NH₄HCO₃, 8 M urea. The yield of recombinants was between 40% and 60%.

SDS Polyacrylamide Disc Gel Electrophoresis—SDS-polyacrylamide disc gel electrophoresis was carried out according to the procedure of Weber et al. (11) with the exception that all samples were not reduced prior to electrophoresis. Reduction was accomplished by boiling for 1 min in a solution containing 1% mercaptoethanol, 0.1% SDS, and 8 M urea. The gels were stained with Coomassie blue.

Amino Acid Analysis—Protein was hydrolyzed in 6 N HCl in vacuo for 22 h and the hydrolysate dried over NaOH. Hydrolysates were analyzed on a Durrum model 500 amino acid analyzer. Tryptophan was determined by hydrolysis in 4 N methanesulfonic acid containing 0.2% 3-(2-aminoethyl)indole (12).

Selective Reduction and Alkylation—In order to determine which interchain disulfide bond or bonds had formed in the hybrid molecules, selective reduction was performed as previously described for hPL (13). Five hundred micrograms (22 nmol) of either recombinant was dissolved in 1 ml of 0.05 M NH₄HCO₃, flushed with N₂, and the protein reduced with 350 nmol of β-mercaptoethanol for 1 h at 37°C. This was followed by the addition of 20 μl of 1 N NaOH containing 1.0 μCi of [14C]iodoacetamide (Amersham, 57 mCi/mmol) and 500 nmol of unlabeled iodoacetamide. The reaction was allowed to proceed for 15 min in the dark at room temperature and was stopped by the addition of 500 nmol of β-mercaptoethanol. [14C]Iodoacetamide which had been incorporated into protein was separated from unincorporated reagent and excess reducing agent by gel filtration on a column (1.5 × 45 cm) of Sephadex G-25 eluted with 0.05 M NH₄HCO₃. The labeled protein was analyzed by SDS-polyacrylamide disc gel electrophoresis. Gels were stained with Coomassie blue and analyzed on a Gilford densitometer at 610 nm or cut into 1-mm slices and extracted with 0.01 M Na barbital buffer, pH 8.6. Portions of the extract were counted in a scintillation counter.

Radioimmunoassays and Radio receptor Assays—The radioimmunoassay for hPL was performed by the double antibody method described earlier (14, 15). Antibodies to purified hPL were produced in guinea pigs. The hGH radioimmunoassay was performed by methods published previously (16–18). hGH was prepared by the lactoperoxidase method (17). The binding of hPL and the recombinants to mammary gland membranes isolated from lactating rabbits (30 days postpartum) was measured by methods described previously (18, 19). The ability of the recombinants to bind to growth hormone receptors isolated from livers of lactating rabbits was measured by the method of Tuszynski and Friesen (20). Competitive binding curves in all assays were analyzed using the logit-log transformation and statistical methods described by Rodbard (21).

Circular Dichroism Spectra—CD measurements were made with a Cary model 60 spectropolarimeter equipped with a Pockels cell. Proteins were dissolved in buffer containing 0.01 M Tris-HCl, 0.01 M KCl, pH 8.0. A path length of 0.1 cm was used for obtaining spectra in the far UV region and 1.0 cm for spectra in the near UV region. Protein concentration was determined from absorbance measurements at 280 nm and by the method of Lowry (9).

FIG. 1. SDS-polyacrylamide disc gel electrophoresis of: 1, reduced hPL; 2, hybrid-hPL; 3, reduced hybrid-hPL; 4, RCAM hPL 1–134; 5, RCAM hGH 141–191. All samples were boiled for 1 min in 0.1% SDS, 8 M urea prior to electrophoresis. Reduction of Samples 1 and 3 was carried out by the addition of β-mercaptoethanol before boiling.

FIG. 2. SDS-polyacrylamide disc gel electrophoresis of hybrid-hPL following reduction and alkylation under nondenaturing conditions. A, Hybrid-hPL alkylated with [14C]iodoacetamide was boiled for 1 min in 0.1% SDS, 8 M urea prior to electrophoresis. B, [14C]-hybrid-hPL was reduced by the addition of β-mercaptoethanol prior to electrophoresis.
RESULTS

Analysis by SDS-polyacrylamide disc gel electrophoresis showed that both hybrids migrated in the position of intact hPL or hGH (Fig. 1). Reduction of the hybrids with β-mercaptoethanol prior to electrophoresis converted them entirely to their component fragments (Fig. 1), indicating that the hybrids contained two fragments joined by one or more disulfide bonds. Amino acid analyses of the hybrids (Table I) were consistent with the amino acid compositions of the constituent fragments. The most notable differences in the amino acid compositions of the 2 hybrid molecules and their parent hormones were in the numbers of residues of aspartic acid, histidine, methionine, and isoleucine.

Since both hPL and hGH contain 3 cysteine residues in their plasmin-generated COOH-terminal fragments, several possible interchain disulfide bonds could have formed. Both native hPL and hGH have disulfide bonds between Cys122 and Cys193 and between Cys82 and Cys150. Since the latter disulfide bond can only be reduced under denaturing conditions, it is possible to reduce and alkylate selectively the COOH-terminal disulfide bond. In order to identify the disulfide bond present in the hPL hybrid, it was reduced and alkylated with [14C]iodoacetamide in the absence of denaturant and analyzed by SDS-polyacrylamide electrophoresis. When the reaction product was applied to the gel without reduction, a single band containing both the protein and radioactivity was detected in the same positions as native hPL (Fig. 2, upper panel). When the same material was boiled in 1% β-mercaptoethanol, 8 M urea prior to electrophoresis, two protein bands were observed (Fig. 2, lower panel), but only the band which corresponded to COOH-terminal Fragment 141-191 contained radioactive alkylating reagent. These results provided evidence that the interchain disulfide formed during recombination was between Cys82 and Cys150. This was confirmed by the observation that the hybrid had no free cysteine residues while the selectively reduced hybrid contained 1.6 residues of carboxymethylcysteine as determined by amino acid analysis.

The immunologic activity of the hybrids was tested in hPL (Fig. 3A) and hGH (Fig. 3B) radioimmunoassays. Based on 50% displacement points, the hPL-hybrid was 65% as active as native hPL in the hPL RIA, but the displacement curves were not parallel. It was nearly completely inactive in the assay with antibodies raised against hGH. hGH-hybrid, on the other hand, was 54% as active as native hGH in the growth hormone RIA, but had almost no activity in the hPL RIA.

In the mammary gland radioreceptor assay (Fig. 4A), both hybrid hormones were 90 to 95% as active as native hPL and produced displacement curves which were nearly identical. In
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filtration on Sephadex G-100 and the major component, which eluted in the same position as intact hPL or hGH, was used for study. Amino acid analysis of this material showed that both NH2-terminal and COOH-terminal fragments were present.

RCAM-hPL hybrid had less than 1% of the immunologic activity of hPL and was totally inactive in both of the membrane receptor binding assays (Table II). RCAM-hGH hybrid was 21% as active as hPL in the mammary gland radioreceptor assay and 11% as potent as hGH in the growth hormone radioreceptor assay. The RCAM hybrids were not stable and precipitated during storage in 0.05 M NH4HCO3 at -20°C.

Fig. 5. Circular dichroism spectra (A) in the region of side chain absorption and (B) in the region of amide bond absorption of hGH (---), PL-hGH (- - -), and hybrid-hGH (-- ---).

Fig. 6. Circular dichroism spectra (A) in the region of side chain absorption and (B) in the region of amide bond absorption for hPL (---), PL-hPL (- - -), and hybrid-hPL (-- --).

The growth hormone radioreceptor assay (Fig. 4B), the activity of hPL hybrid was barely detectable. In contrast, hGH hybrid was 72% as active as native growth hormone in the same assay. The small amount of growth-promoting activity observed for hPL hybrid was not significantly different from that produced by plasmin-modified hPL. The reduced and alkylated NH2-terminal fragments by themselves showed virtually no immunologic or receptor-binding activities.

The circular dichroism of hGH, plasmin-cleaved hGH, and hybrid hGH are shown in Fig. 5. Below 250 nm, all 3 molecules exhibited the same minima at 209 and 222 nm. The values of [θ]MWW at 222 nm for hGH, plasmin-cleaved hGH, and hybrid hGH were 19,550, 19,600, and 19,300, respectively. In the region of side chain absorption the spectra were the same for all three forms of growth hormone, although a slight decrease in intensity occurred at 261 and 269 nm in the plasmin-cleaved hGH and hybrid hGH. The positive ellipticity at 292 nm is identical for hGH and hybrid hGH. The circular dichroic spectra for hPL and hPL hybrid are shown in Fig. 6. Both had large negative ellipticities at 285 nm which were not present in the corresponding hGH derivatives. The intensity at 285 nm was slightly decreased in hybrid-hPL, and the small peak at 277 appeared to be absent. The same differences were also observed with plasmin-cleaved hPL. In the region of amide bond absorption no differences were detected. The values of [θ]MWW at 220 nm for hPL and hybrid hPL were 15,600 and 15,250, respectively.

Hybrids without the Cys57-Cys56 disulfide were prepared from the S-carbamidomethylated fragments as described under "Materials and Methods." They were subjected to gel filtration on Sephadex G-100 and the major component, which eluted in the same position as intact hPL or hGH, was used for study. Amino acid analysis of this material showed that both NH2-terminal and COOH-terminal fragments were present.

RCAM-hPL hybrid had less than 1% of the immunologic activity of hPL and was totally inactive in both of the membrane receptor binding assays (Table II). RCAM-hGH hybrid was 21% as active as hPL in the mammary gland radioreceptor assay and 11% as potent as hGH in the growth hormone radioreceptor assay. The RCAM hybrids were not stable and precipitated during storage in 0.05 M NH4HCO3 at -20°C.

Relative activities were determined from the dose required for 50% displacement and are shown with their 95% confidence limits. RIA, radioimmunoassay; RRA, radioreceptor assay.

<table>
<thead>
<tr>
<th></th>
<th>hPL</th>
<th>hGH</th>
<th>hPL</th>
<th>hGH</th>
</tr>
</thead>
<tbody>
<tr>
<td>RCAM hGH</td>
<td>1-134</td>
<td>141-191</td>
<td>1-134</td>
<td>141-191</td>
</tr>
<tr>
<td>hPL RIA</td>
<td>100</td>
<td>0.09</td>
<td>0.3 (0.1-0.6)</td>
<td>0.3 (0.0-0.4)</td>
</tr>
<tr>
<td>hGH RIA</td>
<td>(0.1-0.3)</td>
<td>100</td>
<td>46 (35-59)</td>
<td>0.5 (0.2-0.9)</td>
</tr>
<tr>
<td>hPL RRA</td>
<td>21 (15-29)</td>
<td>44 (35-59)</td>
<td>11 (5-19)</td>
<td></td>
</tr>
<tr>
<td>hGH RRA</td>
<td>0.2 (0.1-0.3)</td>
<td>0.5 (0.2-0.9)</td>
<td>0.8 (0.4-1.2)</td>
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</table>
DISCUSSION

Amino acid analysis and SDS-polyacrylamide disc gel electrophoresis showed that the hybrid molecules consisted of the NH	extsubscript{2}-terminal fragment of one hormone covalently linked to the COOH-terminal fragment of the other hormone through a disulfide bond. Selective reduction and alkylation of the COOH-terminal disulfide bond of the hPL hybrid without disrupting the interfragment bond indicated that the same disulfide was present in native hPL and the hybrid.

The activities of the hybrids in the hPL and hGH radioimmunoassays suggested that the immunologic determinants recognized by the antibodies were associated with residues 1 to 134. Failure of the hybrids to react significantly in the radioimmunoassay for the hormone which supplied the COOH-terminal fragment implied that few, if any, antibody binding sites were present in residues 141 to 191. Neither of the hybrids were as active as the native hormones. However, it should be noted that plasmin digestion of the hormones reduced their antigenic potency by about 50%, so that with respect to the cleaved molecules, the hybrids regained nearly full immunologic activity. The low immunologic activity of the reduced and alkylated NH	extsubscript{2}-terminal fragments indicated that primary sequence alone was not sufficient for antibody recognition, although the introduction of the carbamidomethyl group on Cys	extsubscript{58} could have produced a conformational change or steric hindrance to antibody binding. Additional structural features, provided by the interaction with the COOH-terminal fragment were necessary for full immunologic activity.

The importance of conformational changes, rather than participation of the COOH-terminal fragment in the antigenic site, is supported by our earlier observation that the hPL-1-134 dimer had markedly enhanced immunologic activity over RCAM-1-134 (7).

Circular dichroism measurements demonstrated further that the structure of each hybrid was similar to that of the hormone from which its NH	extsubscript{2}-terminus was derived. In the region between 270 and 290 nm, hPL-hybrid displayed the native circular dichroic peak characteristic of native hPL. The circular dichroic spectrum of hGH showed a positive peak at 292 nm, a feature which distinguishes it from hPL. The same degree of positive ellipticity at 292 nm was exhibited by hGH-hybrid. Therefore, the environment of the single tryptophan residue, as evidenced by near UV circular dichroic spectra, was dictated by the 1-134 fragments. In addition, the activity at 220 nm indicated that the amount of helical structure present in the hybrids was also determined by the NH	extsubscript{2}-terminal fragments.

The receptor-binding activities of the reduced and alkylated hybrids were in agreement with the values reported by Burstein et al. (22). The fact that the lactogenic potency of reduced and alkylated hGH hybrid was higher than its growth-promoting activity suggested the possibility that the COOH-terminal fragment played a role in determining its biologic specificity. That this was not the case, however, was shown when the disulfide-linked hybrids were measured in the same radioreceptor assays. For each hybrid the lactogenic and growth-promoting activities were nearly identical with those of the hormone which donated its NH	extsubscript{2}-terminal fragment. The fact that hPL-141-191 restored both growth-promoting and lactogenic activity to NH	extsubscript{2}-terminal hGH suggests that the COOH-terminal fragments do not contain information pertaining to biologic specificity. It also provided additional evidence that the receptor binding sites for both hPL and hGH are located within residues 1 to 134. Judging from the low activity of reduced and alkylated NH	extsubscript{2}-terminal fragments, it appears that the COOH-terminal fragments serve to stabilize the active conformation of the molecule.

It should be noted that the hPL-hybrid was not as active as plasmin-modified hPL. Earlier we reported that plasmin-modified hPL was 2 to 3 times more potent than native hPL in the lactogenic receptor assay. The reduced activity of hybrid-hPL compared to plasmin-modified hPL could be due either to the procedure used for recombination or to conformational changes induced by the presence of COOH-terminal hGH. The former explanation is supported by recent experiments which indicate that reduction and reoxidation of plasmin-modified hPL results in a similar decrease in lactogenic binding activity.

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REFERENCES
