Selective Modification of Recombinant Bovine Placental Lactogen by Site-directed Mutagenesis at Its C Terminus

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Five recombinant analogues of bovine placental lactogen (bPL) ((bPL(S184H), bPL(S187A), bPL(S187F), bPL(T188F), bPL(T188F,I190F)) were prepared, expressed in Escherichia coli, and purified to homogeneity. Circular dichroism analysis revealed no or minor structural changes, except in bPL(T188F,I190F). Binding and biological activities of bPL(T188F,I190F) were almost completely abolished, whereas bPL analogues mutated at position 187 retained their full activity. Point mutation T188F resulted in selective modification; binding to somatogenic receptors, their extracellular domains (ECDs), and to bPLR in the endometrium as well as somatogenic receptor-mediated biological activities were reduced or abolished, whereas binding to lactogenic receptors, their ECDs, and subsequent biological activity was fully or almost fully retained. This selective modification most likely results from a steric hindrance induced by a bulky Phe-188 chain of bPL which interacts with the Arg-43 of the human or Leu-43 of the non-human GHRs. Point mutation S184H abolished the interaction with hGHR, most likely due to the unfavorable charge-charge interaction, possibly accompanied by steric hindrance between Arg-43 of the receptor and the newly introduced His-184 and possible interference with the putative interaction between the alkylation point of Thr-188 and Lys-185 of bPL with Trp-104 of hGHR. In contrast, bPL(S184H) retained its capacity to interact with nonhuman GHRs. Decrease in the biological activity of bPL(S184H) was also observed in two lactogenic receptor-mediated bioassays most likely due to the elimination of the intermolecular hydrogen bond of Ser-184 with a side chain of Tyr-127, which appears in all lactogenic receptors.

Bovine placental lactogen (bPL) has been purified from term placental homogenates and from isolated secretory granules obtained from binucleate cells of fetal cotyledon. The native 31 to 33 kDa bPL has at least five isoelectric variants which are in part due to heterogeneity of the attached oligosaccharides, and to as yet unidentified modifications. The gene for bPL has been cloned and expressed with high efficiency in Escherichia coli and the recombinant bPL has been purified to homogeneity (3). The predicted mature bPL has 200 residues and the primary sequence exhibits 50% and 23% homology to bovine prolactin (bPRL) and growth hormone (bGH), respectively (3). In comparison with bGH and bGH, bPL has 12–13 additional amino acids at the N-terminal portion of the molecule and, in common with mammalian PRLs, it has a third disulfide bond located in this N-terminal region. Deglycosylation of native bPL had no effect on PRL-like mitogenic activity in an Nb2 lymphoma cell proliferation assay in which bPL was equally potent to hGH, bPRL, and ovine (o)PRL, and exhibited only slightly reduced binding to bGH receptors (4). Recombinant bPL was also equally potent to hGH or oGH in somatogenic receptor-mediated 3T3-L1 or 3T3-F442A preadipocyte bioassays (5, 6). However, in a homologous lactogenic receptor-mediated bioassay using bovine mammary gland explants, we found that the native bPL is also as potent as hGH or bPRL (7). Binding experiments to various microsomal fractions revealed that bPL binds with high affinity to prolactin receptors and to somatogenic receptors (2, 4, 5). In addition, we have documented that bPL binds also to unique receptors in bovine endometrium (8). Thus bPL is an unique hormone which may transduce its activity through three different receptors.

Structure-function relationship studies of bPL have been conducted in our laboratory by successive truncation of its C-terminal domain (9, 10). Assuming structural similarity to porcine GH (11) these mutations were aimed to remove amino acids beyond or at the beginning of the putative first α-helix and vice versa. Information obtained from mutated analogues of hGH indicates that not only the N-terminal domain (12, 13, 17), but also the C-terminal domain and the non-helical sequence intervening between the first and the second α-helix participate in receptor binding (18). Publications concerning mutation of this domain in hGH (18–20) implicates its importance in the binding to somatogenic receptors as well as lactogenic receptors (21). In the present work several mutations in an analogous region of bPL were incorporated. The rationale behind creating these mutations was based mainly on the findings using ala- nine scanning mutagenesis of hGH (18–20) and the structural analysis of hGH:hGHR-ECD complex (22). One of the most dramatic changes was obtained with the E174A mutation of hGH which resulted in a 4-fold increase in affinity toward somatogenic receptor with a simultaneous 356-fold decrease in

*This research was supported by USA-Israel Binational Agricultural and Development Fund (BARD) Grant US-2109–92R. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡The abbreviations used are: PL, placental lactogen; PRLR-ECD, prolactin receptor extracellular domain; GH, growth hormone; PRL, prolactin; h, human; b, bovine; o, ovine; r, rat; rb, rabbit; PAGE, polyacrylamide gel electrophoresis.
affinity for lactogenic receptor, and the D171A mutation which acted in the opposite way. Asp-171 and Trp-175 of hGH have been found to participate in the formation of hydrogen bonds with Arg-43 of hGHR-ECD, stabilizing the complex (22). These mutations are found in a portion of the sequence DTVET (171–175). The corresponding amino acids of bPL are most likely SKIST (184–188). To evaluate the importance of these amino acids in bPL action, we prepared five analogues with changes in these or neighboring residues. In most cases we mutated the respective residues to phenylalanine in order to increase the hydrophobicity and to introduce a large side chain. In one case (position 184), we preferred mutation to histidine since this amino acid occupies the corresponding position in all nonhuman GHs (23).

**EXPERIMENTAL PROCEDURES**

Materials—Recombinant bPL was prepared as described previously (9) and recombinant hGH was obtained from Biotechnology General Inc. (Israel). Recombinant non-glycosylated rabbit (rb), bovine (b), and rat (r) PRLR-ECD and human (h) GHR-ECD were prepared as described previously (24–27). Carrier-free Na<sup>131I</sup> was purchased from Du Pont NEN. Molecular weight (M<sub>r</sub>) markers for gel electrophoresis, RPMI 1640 medium, lysozyme, Triton X-100, horse myoglobin, and bovine serum albumin (radioimmunoassay grade), were obtained from Sigma. SDS-PAGE reagents and Protein Assay Kit were purchased from Bio-Rad. Superdex<sup>TM</sup> 75 HR 10/30 column and Q-Sepharose (fast flow) were purchased from Pharmacia LKB Biotechnology AB (Uppsala, Sweden). All other chemicals were of analytical grade.

Construction of bPL Analogues Expression Vectors—Synthetic genes for each bPL analogue were constructed using polymerase chain technology. Synthetic oligonucleotides (primers) were used to generate a double-stranded DNA from a template, pMON3401 (28), for subcloning. An NcoI site was created with a forward primer at the 5′ end of the gene, which also added an initiator methionine codon immediately upstream of the first mature codon (alanyle), and a HindIII site was created at the 3′ end of the gene with a reverse “mutant” primer. The reverse mutant primers encoded the mutation(s) of interest and a termination codon as part of the gene, which also added an initiator methionine codon immediately upstream of the first mature codon (alanyle), and a HindIII site was created at the 3′ end of the gene with a reverse “mutant” primer. The reverse mutant primers encoded the mutation(s) of interest and a termination codon as part of the gene.

Expression, Refolding, and Purification of bPL Analogues—Transformed E. coli cells (250 ml) were grown in LB media at 37°C in 2-liter flasks to an A<sub>600</sub> of 0.9, after which nalidixic acid (50 mg/liter) was added. Cells were grown for an additional 4 h and then harvested by 10-min centrifugation at 10,000 × g and frozen. Over 95% of bPL protein was found in the inclusion bodies which were prepared as described previously (9). The inclusion body pellet containing the bPL analogues was solubilized in 600 ml of 4.5 M urea buffered with 40 mM Tris base. The pH was increased to 11.3 with NaOH, cysteine was added to 0.1 M, and the clear solution was stirred at 4°C for 48 h and then dialyzed for 48 h against 4 × 10 liters of 10 mM Tris-HCl, pH 9. The solution was loaded at 200 ml/h onto a Q-Sepharose column (2.6 × 7 cm), pre-equilibrated with 10 mM Tris-HCl, pH 9.0, at 4°C. Elution was carried out using a discontinuous NaCl gradient in the same buffer at a rate of 100 ml/h, and 5-ml fractions were collected. Protein concentration was determined by absorbance at 280 nm, and monomer content by gel filtration chromatography on a Superdex<sup>TM</sup> 75 column.

SDS-Polyacrylamide Gel Electrophoresis—SDS-PAGE was carried out according to Laemmli (29) using 10 or 12% gels. Gels were stained with Coomassie Brilliant Blue R.

Biochemical Analysis of Monomer Content and Complex Formation—High performance liquid chromatography gel filtration chromatography on a Superdex<sup>TM</sup> 75 HR 10/30 column, using 25 mM Tris-HCl buffer, pH 8, containing 150 mM NaCl and 10 mM MgCl<sub>2</sub> (TNM buffer), was performed on 200-μl aliquots of Q-Sepharose column-eluted fractions, freeze-dried samples dissolved in H<sub>2</sub>O, or complexes between the soluble recombinant GHR- or PRLR-ECDs and various bPL analogues, using methods detailed previously (24–26).

Circular Dichroic (CD) Spectra—CD spectra were collected at 4°C in a Jasco J-500C spectropolarimeter in either 0.2- or 0.5-mm cylindrical cells. The spectropolarimeter was routinely calibrated with α-cyclo-T(+) camphor sulfonic acid at 290 nm. The CD spectra (average of 4 scans) were baseline corrected and converted to mean residue molar ellipticity ([θ]) and analyzed by a least square fitting procedure (30). Additionally, the α-helix content was estimated by the magnitude of [θ] at 222 nm (31). The absorbance was used to estimate the protein concentration for CD analysis using the method of Gill and von Hippel (32).

**RESULTS**

Preparation of bPL Analogues—Five bPL analogues were produced in E. coli and purified by ion-exchange chromatography on a Q-Sepharose column. The main protein peak eluted with 150 mM NaCl contained the pure monomeric bPL analogues, as evidenced by gel filtration on a Superdex column conducted under non-denaturing conditions and by SDS-PAGE under reducing and non-reducing conditions (not shown). These fractions were pooled, dialyzed against 0.05% NaHCO<sub>3</sub>, freeze-dried, and stored at −20°C.

Structural Evaluation—The CD spectrum of bPL is generally consistent with the roughly 50% α-helix determined previously (9). The CD analysis for bPL and several analogues were repeated to estimate the variability of the method (Table I). The overall variability was about ±4% helix, or values between 51% helix and 59% helix are experimentally indistinguishable from native bPL (55 ± 4, mean ± 1 standard deviation). Analogues bPL(S184H), bPL(S187F), and bPL(S188F) are experimentally identical to native bPL. Analogue bPL(T188F,I190F) has roughly half the helical content of native bPL. The two Phe substitutions in the C-terminal region could disrupt the local protein structure, but also have a large effect in overall secondary structure. Analogue bPL(S187A) has a slight decrease in helix content, which causes minor secondary structure reorganization.

Binding Experiments—Binding to the soluble PRLR-ECDs, hGHR-ECD, bovine liver, and bovine endometrium microsomal fractions and to intact IM-9 lymphocytes and Nb<sub>6</sub> lymphoma cell homogenates was carried out as described previously (8–10, 24–26).

In Vitro Bioassays—Five in vitro bioassays were used as described previously: Nb<sub>6</sub>-1IC lymphoma cell proliferation bioassay (33), β-casein expression in a HC11 mouse mammary-gland derived cell line (25), o-casein production in rabbit mammary gland explants (34), anti-maternal somatogenic receptor-mediated activity in 3T3-F442A rat preadipocytes (6), and IGF-I secretion from a primary culture of rat hepatocytes (16).

<table>
<thead>
<tr>
<th>Analogue</th>
<th>%α helix</th>
<th>Yang method for two-dimensional analysis&lt;sup&gt;a&lt;/sup&gt; (analysis from 240 to 195 nm)</th>
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<tr>
<td>Unmodified</td>
<td>53</td>
<td>54</td>
</tr>
<tr>
<td>S184H</td>
<td>51</td>
<td>50</td>
</tr>
<tr>
<td>S187A</td>
<td>49</td>
<td>46</td>
</tr>
<tr>
<td>S187F</td>
<td>61</td>
<td>58</td>
</tr>
<tr>
<td>S188F</td>
<td>4</td>
<td>2</td>
</tr>
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</table>

<sup>a</sup> Ref. 30.
<sup>b</sup> Ref. 31.
the analogue bPL(T188F,I190F) almost completely lost its ability to compete with the ligand in all four assays. In contrast, the analogue bPL(T188F) completely lost its ability to compete for binding to hGHR-ECD, whereas its ability to compete for binding to rPRLR-ECDs was not affected (bovine) or 3–12-fold reduced (rat and rabbit). Similarly, the ability of bPL(S184H) to compete for binding to hGHR-ECD or to rbPRL-ECD was reduced over 100- and 3-fold, respectively, whereas its ability to bind to other PRLR-ECDs was unchanged or only slightly affected. Almost identical results were obtained when determining the effect of mutations on the binding to somatogenic and lactogenic receptors in intact cells or in microsomal fractions (Fig. 2). Again, the ability of bPL(T188F,I190F) to bind in all four assays was lost or drastically reduced, whereas the ability of bPL(T188F) to compete for binding was not affected in Nb2 cells, was reduced in bovine liver and even more in bovine endometrium microsomal fractions and was lost in IM-9 human lymphocytes. In contrast, the binding of bPL(S184H) was lost in IM-9 human lymphocytes but was retained in all other assays. The activity of the other bPL analogues were unchanged.

Complex Formation—The stoichiometry of complex formation between various bPL analogues and soluble hGHR-ECD was tested at three hormone:hGHR-ECD molar ratios. In that experiment the concentration of the latter was kept constant, whereas the concentrations of the respective hormones were increased (Fig. 3). Bovine PRL formed a 1:2 complex with hGHR-ECD, confirming our previous results that were based both on gel filtration and SDS-PAGE experiments (10). The same results, obtained with bPL(S187A) and bPL(S187F), are not shown. Almost identical results were obtained with bPL(S184H) as documented by similar retention time values and peak ratios (Fig. 3B), despite the drastically reduced binding ability of this analogue (Fig. 1A). These contradictory results may be most likely attributed to the fact that binding experiments were performed in nanomolar, whereas the gel filtration experiments in micromolar concentrations. Since the K_d of the bPL:hGHR-ECD interaction is 0.6 nM (10), even at ~100-fold increase in K_d value in bPL(S184) still allowed complex formation at micromolar concentrations. In contrast, bPL(T188F,I190F) did not form any complex (Fig. 3D), whereas bPL(T188F) was capable of forming only a weak 1:1 complex that underwent partial dissociation in the course of chromatography (Fig. 3C). At 1:1 bPL(T188F):ECD ratio and 15 μM concentration of both reagents a right skewed peak with retention time of 18.78 min (that corresponds to molecular mass of the 1:1 complex) was found. Increasing the analogue:hGHR-ECD ratio to 2:1 and 5:1, with a corresponding dilution of the complex, shifted this peak, respectively, to RT of 19.04 and 19.72 min. Our interpretation of these results is that due to dilution of the complex from 15 to 3 μM it underwent partial dissociation in the course of chromatography. Lack of clear separation between the complex and the excess of the hGHR-ECD supports this interpretation.

Complex formation between bPL analogues and rabbit and rPRLR-ECDs was monitored by gel chromatography using constant hormone and variable ECD concentrations (Figs. 4 and 5). These results showed that increase in the ECD to hormone ratio from 1:1 to 2:1 (Fig. 4A and 5A) resulted in doubling of the peak area, thus confirming our previous observations that in both cases bPL forms a 1:2 complex with each R-ECD (27, 37). Increase of the ECD:analogue molar ratio to 3:1 did not further increase the size of the complex and the excess of the ECDs were observed. Analysis of the retention times of the eluted

![Figure 1](link)

**Fig. 1.** Binding of 125I-hGH to hGHR-ECD or bPRLR-ECD and binding of 125I-bP to rbPRLR-ECD or rPRLR-ECD. Competitive binding was determined by simultaneous addition of bP (●), bPL(S184H) (△), bPL(S187A) (■), bPL(S187F) (○), and bPL(T188F,I190F) (□). Non-specific binding was obtained by the addition of 2000 ng/ml hGH or bP, respectively. The actual specific binding was 22, 12, 27, 6, and 17%, respectively.

**Table II**

<table>
<thead>
<tr>
<th>Parameter tested</th>
<th>bP analogues</th>
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<tr>
<td></td>
<td>S184H</td>
</tr>
<tr>
<td>Binding to hGHR-ECD</td>
<td>0.9</td>
</tr>
<tr>
<td>Binding to rPRLR-ECD</td>
<td>30 ± 5</td>
</tr>
<tr>
<td>Binding to rPRLR-ECD</td>
<td>77 ± 4</td>
</tr>
<tr>
<td>Binding to hGHR-ECD</td>
<td>65 ± 4</td>
</tr>
<tr>
<td>Binding to IM-9 cells</td>
<td>0.5</td>
</tr>
<tr>
<td>Binding to Nb2 cells</td>
<td>0.12 ± 2</td>
</tr>
<tr>
<td>Binding to liver microsomes</td>
<td>120 ± 12</td>
</tr>
<tr>
<td>Binding to endometrium microsomes</td>
<td>166 ± 40</td>
</tr>
<tr>
<td>Nb2 cell proliferation</td>
<td>52 ± 11</td>
</tr>
<tr>
<td>β-casein synthesis in HC11 cells</td>
<td>32 ± 7</td>
</tr>
<tr>
<td>α-casein synthesis in rbMGα explants</td>
<td>100 ± 31</td>
</tr>
<tr>
<td>IGF-I secretion in rat hepatocytes</td>
<td>90 ± 6</td>
</tr>
<tr>
<td>Anti-mitogenic activity in 3T3-F442A preadipocytes</td>
<td>110 ± 18</td>
</tr>
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</table>

*a* MG, mammary gland.

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**Table II** Comparison of bP and its analogues

The activities of bP analogues are presented relative to bP, which was taken as 100. Each experiment was performed twice and the results are given as a mean ± S.E. The results of the binding experiments and the bioassays were, respectively, calculated from their IC_{50} and ED_{50} values. The S.E. of bP varied in different assays between 5 and 11% of the respective mean. For values less than 1 the S.E. were not calculated. To facilitate the comparison of the IC_{50} values the following K_a values were calculated from the respective binding assays using the same hormone as a ligand and a displace: hGH:hGHR-ECD, 0.6 nM; bPL:rbPRLR-ECD, 0.23 nM; bPL:rPRLR-ECD, 3.37 μM; hGH:bPRLR-ECD, 2.70 μM (bP has approximately 20-fold lower affinity); hGH:hGHR in IM-9 cells, 0.29 nM; hGH:PRLR in Nb2 cells, 0.27 nM; bGH:bGHR in liver microsomes, 0.1 nM; and bPL:bPRLR in bovine endometrium, 0.035 nM.
peaks indicated, however, that at initial 0.5:1 and 1:1 bPL: PRLR-ECD ratios a 1:2 complex and excess bPL were observed rather than a 1:1 complex. The smaller size of the bPL peak results from its almost 3-fold lower specific extinction coefficient at 280 nm. The small difference in retention times in experiments with rabbit and rPRLR-ECDs originates from using two columns with slightly different bead volumes and thus is irrelevant to an interpretation of the results. Three bPL analogues, bPL(S184H), bPL(S187A), and bPL(S187F), not shown in the figure yielded results identical to those of the unmodified hormone. In contrast, bPL(T188F) and bPL(T188F,I190F) retained their ability to form a 1:2 complex with rPRLR-ECD (Fig. 5, A and B) but formed only a 1:1 complex with rPRLR-ECD (Fig. 4, B and C).

We have recently shown by gel filtration experiments that at 1.6 \( \mu \)M concentration of both reagents of bPL forms a weak 1:1 complex with bPRLR-ECD. Lowering the reagent concentrations by 2-16-fold led to its progressive dissociation (26). Similar results performed at 3.3 \( \mu \)M concentration were obtained in the present work with bPL(S184H), bPL(S187A), bPL(S187F), and bPL(T188F), although the dissociation of the later occurred already at relatively higher absolute concentrations (not shown). Since \( \text{I}^{251} \)-bPRL binds very poorly in this system, and the \( K_d \) of the bPL-bPRRL complex could not be calculated from homologous binding experiments, its affinity toward bPRLR-ECD was evaluated from the IC\( _{50} \) values using \( \text{I}^{251} \)-hGH as a ligand. The \( K_d \) of the hGH:bPRLR-ECD calculated from the data in Fig. 1 was 2.7 \( \mu \)M in agreement to the previously reported value of 2.07 \( \mu \)M (26), and the relative IC\( _{50} \) of bPL was 20-fold higher than that of hGH. These results fully explain and support our interpretation of gel filtration experiments. In contrast, no complexes could be detected between bPL(T188F,I190F) and bPRLR-ECD, even at a 3-fold excess of the latter (not shown).

Bioassays—The biological activity of the bPL analogues was determined in five in vitro bioassays in which hormone activity is mediated by lactogenic (Nb\(_2\), HC11, and explants of rabbit mammary gland) and somatogenic (3T3-F442A preadipocytes and rat hepatocytes) types of receptors (Fig. 6). The results clearly indicate that the bioactivities of bPL(S187A) and bPL(S187F) remained essentially unchanged in all bioassays, whereas that of bPL(T188F,I190F) was drastically reduced (Nb\(_2\), HC11), or almost completely abolished (rat hepatocytes, 3T3-F442A, rabbit mammary gland explants). Yet bPL(T188F) retained its activity in Nb\(_2\), and rabbit mammary explant lactogenic receptor-mediated bioassays, lost ~60% of its activity in HC11 bioassay, whereas its activity in the two somatogenic receptor-mediated bioassays was reduced approximately 12-20-fold. In contrast, bPL(S184H) retained its full biological activity in both bioassays mediated through somatogenic receptors and in the rabbit mammary gland explants, whereas its biological activity in Nb\(_2\) and HC11 cells decreased 2-3-fold.

**DISCUSSION**

CD analysis of the bPL analogues revealed preservation of their secondary and, most likely, tertiary structure with the exception of the double-mutated bPL(T188F,I190F) analogue. Thus, any functional changes resulting from these mutations probably do not originate from an overall structural change but rather from disruption of local hormone-receptor contacts. In contrast, the overall \( \alpha \)-helix content in bPL(T188F,I190F) and exposure of Tyr-189 to the solvent were reduced and the environment of both Trp was changed. In hGH, the side chain of the
corresponding residue (Leu-177) is completely buried as an integral part of the four-helix bundle core (22). These differences most likely account for the altered structure of this analogue that results in almost complete loss of receptor binding and biological activities (Table II). Yet this drastically modified analogue could bind to respective receptors and exhibit some biological activity when its concentration was increased 10–1000-fold over that of unmodified bPL.

Modification of Ser-187 to either Ala or Phe minimally affects its binding properties or biological activities. Ser-187 corresponds to Glu-174 in hGH (Table III), whose mutation to Ala dramatically changes its somatogenic/lactogenic receptor specificity, mainly by reducing the binding to lactogenic receptor (20). This difference may be attributed to the fact that lactogenic effects of hGH are strongly dependent on Zn$^{2+}$ and the Glu-174 of hGH is part of the Zn$^{2+}$ binding site (20).

Two other mutations resulted, however, in specific modifications of binding properties and biological activity (Table II). Analogue bPL(T188F) lost the ability to bind to human somatogenic receptors, to bovine liver microsomal fraction, and most of its ability to bind to the endometrial microsomal fraction which most likely contains unique bPL receptors (8). By comparison, its ability to bind to lactogenic receptors was either unchanged (intact Nb$_2$ cells, bovine PRLR-ECD) or reduced (rat and rbPRLR-ECDs). The reduced ability of bPL(T188F) to bind to human somatogenic receptor was also reflected by its inability to form a 1:2 complex with hGHR-ECD and the formation of a weak 1:1 complex only. However, the ability of this analogue to form complexes with ECDs of various PRLRs was unchanged or only slightly modified. In parallel, its somatogenic receptor-mediated biological activity was reduced 20-fold in rat hepatocytes and 12-fold in mouse 3T3-F442A preadipocytes, whereas its lactogenic receptor-mediated activity was either unaffected (Nb$_2$-11C cells and rabbit mammary gland explants) or 2.5-fold reduced (HC11 cells).

Selective modification also occurred with the S184H mutation leading to a 100–200-fold decrease in binding to human, but not other, somatogenic receptors. Conversely, its ability to bind to lactogenic or bPL receptors remained unchanged (bovine endometrial, intact Nb$_2$-11C cells, and rabbit mammary gland explants) or 2.5-fold reduced (rbPRLR-ECD).

Selective modification also occurred with the S184H mutation leading to a 100–200-fold decrease in binding to human, but not other, somatogenic receptors. Conversely, its ability to bind to lactogenic or bPL receptors remained unchanged (bovine endometrial, intact Nb$_2$-11C cells, and rabbit mammary gland explants) or 2.5-fold reduced (rbPRLR-ECD). Yet, despite the drastically reduced ability to bind to intact IM-9 cells or hGHR-ECD, bPL(S184H) at micromolar concentration was capable of forming 1:2 complexes with hGHR-ECD, as evidenced by gel filtration experiments. At the same time, the somatogenic rat or mouse receptor-mediated biopotency of bPL(S184H) was unchanged. Some decrease, however, was observed in the biological activity mediated through lactogenic receptors in Nb$_2$-11C or HC11 cells. Reduced biological activity of bPL(S184H) in Nb$_2$-11C cells was not, however, paralleled by a corresponding change in binding to intact cells, indicating that binding properties of hormones or their analogues are not always indicative of biological potency. When interpreting these results, one
needs to bear in mind the fact that the gel filtration experiments were performed at reagent concentrations (ECD and hormone) that were 100-1000-fold higher than those used in competition binding experiments. Since dilution of the complexes to concentrations close to their K_d values obviously leads to dissociation, results of our previous dimerization experiments should be interpreted with caution (24, 25, 37–40), as should the fact that proteins and ECDs. In IM-9 cells and hGHR-ECD the different analogues yielded almost identical results, but bPL(T188F) is selective and leads to a loss in its somatogenic activity only. In contrast to bPL(T188F), bPL(S184H) retained its ability to bind to nonhuman somatogenic receptors and to exhibit full somatogenic receptor-mediated biological activity in rat hepatocytes and 3T3 F442A preadipocytes as shown by curves. bPL (●), bPL(S184H) (●), bPL(S187A) (●), bPL(S187F) (●), bPL(T188F) (○), and bPL(T188F,1190F) (□). IGF-I secretion from a primary culture of rat hepatocytes and α-casein production in rabbit mammary gland explant are shown by bars; bPL (□), bPL(S184H) ( ), bPL(S187A) (■), bPL(S187F) ( ), bPL(T188F) ( ), bPL(T188F,1190F) (□). The original blot of bPL ( ), bPL(S184H) ( ), bPL(S187A) (■), bPL(S187F) ( ), bPL(T188F) ( ), and bPL(T188F,1190F) (□). Table III: Sequence alignment of hGH, bGH, and bPL (residues 171-191, hGH numbering).

<table>
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<tr>
<th></th>
<th>hGH 171</th>
<th>bGH 171</th>
<th>bPL 184</th>
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<tr>
<td>hGH</td>
<td>DKVETFLAV</td>
<td>QCRS.VEGSG</td>
<td>F 191</td>
</tr>
<tr>
<td>bGH</td>
<td>HKTETYLKV</td>
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<td>F 192</td>
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<tr>
<td>bPL</td>
<td>SKISTYINL</td>
<td>KCR... FTPC</td>
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Although the three-dimensional structure of bPL has yet to be resolved, sequence comparison suggests high homology to hGH, in which both Thr-175 and Asp-171 are located at the outer surface of the fourth α-helix (22). The corresponding amino acids in bPL are most likely Thr-188 and Ser-184. In hGH, Thr-175 O^2 and Asp-171 O^2 form intermolecular hydrogen bonds with Arg-43 N^1 and Arg-43 N^2 of hGH-ECD (22). These contacts, and in particular that of Thr-175, seem to be most critical for high-affinity complex formation, as exemplified by the fact that its mutation to Ala reduced the affinity by over 2 kcal/mol and that it was highly (75%) conserved after sorting for high-affinity mutants that bind to hGH-ECD (43, 44). Our present results support the hypothesis that Thr-188 of bPL indeed occupies the Thr-175 position of hGH. Its mutation to a bulky Phe residue most likely interferes with complex formation, since bPL(T188F) retains its biological activity in Nb2 cells and rabbit mammary gland explants which are mediated through lactogenic receptors, we conclude that the buried area is most likely large enough to contain the benzene ring of Phe. These structural features explain why the modification of bPL(T188F) is selective and leads to a loss in its somatogenic activity only.
PRLRs, but it is unclear whether Ser-184 of bPL also forms a parallel hydrogen bond. Results concerning the lactogenic receptor-mediated biological activity of bPL(S184H) were not consistent. Whereas in Nb and HC11 cells an 2–3-fold decrease was observed, the activity in rabbit mammary gland explants was unchanged. This reduced activity was not always paralleled by reduced affinity toward soluble or membrane-embedded PRLRs. These results are difficult to explain and may be related to minor structural differences between various PRLRs, as exemplified by our studies with their soluble ECDs (24–27, 37).

REFERENCES
35. Deleted in proof.
36. Deleted in proof.