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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 alkyl portion 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)[1] has been purified from term placental homogenates (1) and from isolated secretory granules obtained from binucleate cells of fetal cotyledon (2). 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 hGH, 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 Nb_2 lymphoma cell proliferation assay in which bPL was equally potent to human (h)GH, 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 N-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. Results of these studies (9, 10) indicated that similarly to hGH (12–16), bPL can be selectively modified so that particular biological activities are changed while others remain relatively unaffected.

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), implicate its importance in the binding to somatogenic receptors as well to 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 alanine 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

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[1] 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 DKVET (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).

TABLE I
Estimated secondary structure for bPL analogues

Analogue	% α helix	Yang method for two-dimensional analysis[a] (analysis from 240 to 195 nm)			
bPL analogue	MRME$_{222}$[b]	% α	% β	% Turn	% Random
Unmodified	53	54	12	3	31
S184H	51	50	16	1	33
S187A	49	46	20	2	32
S187F	61	58	4	2	35
T188F,I190F	26	26	39	3	31
T188F	50	52	10	8	30

[a] Ref. 30.
[b] Ref. 31.

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[^{125}I] was purchased from DuPont NEN. Molecular weight (M_r) 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™ 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 *Nco*I site was created with a forward primer at the 5′ end of the gene, which also added an initiator methionine codon immediately upstream to the first mature codon (alanine), and a *Hin*dIII 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 *Hin*dIII site. The polymerase chain reaction products were purified using a Wizard™ polymerase chain reaction kit (Promega, Madison, WI) and digested with *Nco*I and *Hin*dIII restriction enzymes prior to ligation to parental vector pMON3401 using T$_4$ DNA ligase. The resulting plasmids were sequenced using a Sequenase™ DNA sequencing kit (U. S. Biochemical Corp.) and the positive constructs were passaged through *E. coli* strain LE392 prior to tranformation of MON105 (28).

Expression, Refolding, and Purification of bPL Analogues—Transfected *E. coli* cells (250 ml) were grown in LB media at 37 °C in 2-liter flasks to an A_{600} 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 mM, 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 then 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® 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.

Determination of Monomer Content and Complex Formation—High performance liquid chromatography gel filtration chromatography on a Superdex® 75 HR 10/30 column, using 25 mM Tris-HCl buffer, pH 8, containing 150 mM NaCl and 10 mM MgCl$_2$ (TNM buffer), was performed on 200-μl aliquots of Q-Sepharose column-eluted fractions, freeze-dried samples dissolved in H$_2$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 D-10-(+)-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). A sample of myoglobin was analyzed in parallel with the bPL samples and was found to agree with literature values (~80% helix, Ref. 30).

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$_2$ 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$_2$-11C lymphoma cell proliferation bioassay (33), β-casein expression in a HC11 mouse mammary-gland derived cell line (25), α-casein production in rabbit mammary gland explants (34), antimitogenic somatogenic receptor-mediated activity in 3T3-F442A rat preadipocytes (6), and IGF-I secretion from a primary culture of rat hepatocytes (16).

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 nondenaturing conditions and by SDS-PAGE under reducing and nonreducing conditions (not shown). These fractions were pooled, dialyzed against 0.05% NaHCO$_3$, 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 of bPL analogues to soluble hGHR-ECD or PRLR-ECDs from different species revealed that the mutations selectively modify binding (Fig. 1). In general the effect of mutations was tested by comparison of displacement curves and calculation of the respective IC$_{50}$ values. In order to facilitate the comparison between different experiments the respective K_d values were calculated from Scatchard plots using the same hormone as a ligand and a displacer (see footnote to Table II). Whereas the binding of analogues bPL(S187A) and bPL(S187F) were not or only slightly affected, 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 PRLR-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 Nb$_2$ 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.

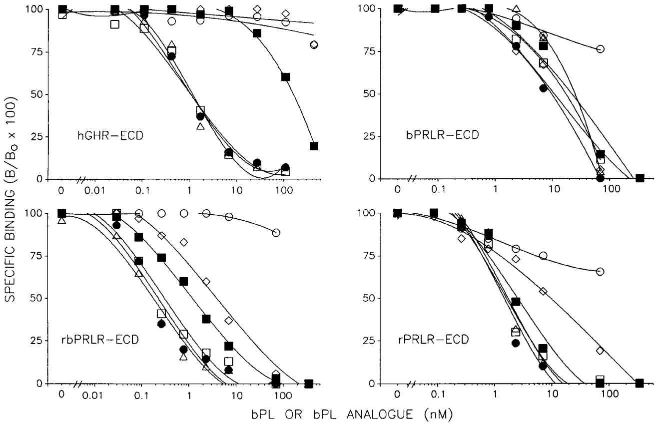

FIG. 1. **Binding of ^{125}I-hGH to hGHR-ECD or bPRLR-ECD and binding of ^{125}I-bPL to rbPRLR-ECD or rPRLR-ECD.** Competitive binding was determined by simultaneous addition of bPL (●), bPL(S184H) (■), bPL(S187A) (□), bPL(S187F) (△), bPL(T188F) (◇), and bPL(T188F,I190F) (○). Nonspecific binding was obtained by the addition of 2000 ng/tube hGH or bPL, respectively. The actual specific binding was 22, 12, 27.6, and 17%, respectively.

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 PL 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. 3*B*), despite the drastically reduced binding ability of this analogue (Fig. 1*A*). 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. 3*D*), whereas bPL(T188F) was capable of forming only a weak 1:1 complex that underwent partial dissociation in the course of chromatography (Fig. 3*C*). 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. 4*A* and 5*A*) 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

TABLE II
Comparison of bPL and its analogues

The activities of bPL analogues are presented relative to bPL, which was taken as 100. Each experiment was performed two to four times and therefore the values 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 bPL 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_d values were calculated from the respective binding assays using the same hormone as a ligand and a displacer: hGH:hGHR-ECD, 0.6 nM; bPL:rbPRLR-ECD, 0.23 nM; bPL:rPRLR-ECD, 3.37 nM; hGH:bPRLR-ECD, 2.70 nM (bPL has approximately 20-fold lower affinity); hGH:hGHR in IM9 cells, 0.29 nM; hGH:PRLR in Nb$_2$ cells, 0.27 nM; bGH:bGHR in liver microsomes, 0.1 nM; and bPL:bPLR in bovine endometrium, 0.035 nM.

Parameter tested	bPL analogues				
	S184H	S187A	S187F	T188F	T188F,I190F
Binding to hGHR-ECD	0.9	100 ± 11	100 ± 12	0.1	0
Binding to rbPRLR-ECD	30 ± 5	86 ± 7	125 ± 15	8 ± 2	>0.1
Binding to rPRLR-ECD	77 ± 4	100 ± 10	100 ± 9	30 ± 7	0.2
Binding to bPRLR-ECD	65 ± 4	80 ± 6	65 ± 5	100 ± 3	1.0
Binding to IM-9 cells	0.5	135 ± 30	135 ± 25	0.1	0
Binding to Nb$_2$ cells	102 ± 22	119 ± 24	150 ± 13	105 ± 12	6 ± 1
Binding to liver microsomes	120 ± 12	100 ± 6	120 ± 10	20 ± 3	>0.1
Binding to endometrium microsomes	166 ± 40	90 ± 20	100 ± 20	3 ± 2	>0.1
Nb$_2$ cell proliferation	52 ± 11	160 ± 20	166 ± 19	155 ± 18	6 ± 1
β-Casein synthesis in HC11 cells	32 ± 7	121 ± 5	93 ± 10	38 ± 2	4 ± 3
α-Casein synthesis in rbMG^a explants	100 ± 31	71 ± 28	71 ± 8	77 ± 15	5 ± 3
IGF-I secretion in rat hepatocytes	90 ± 6	75 ± 5	95 ± 5	5 ± 1	0.5
Anti-mitogenic activity in 3T3-F442A preadipocytes	110 ± 18	120 ± 24	80 ± 15	8 ± 1	0.5

[a] MG, mammary gland.

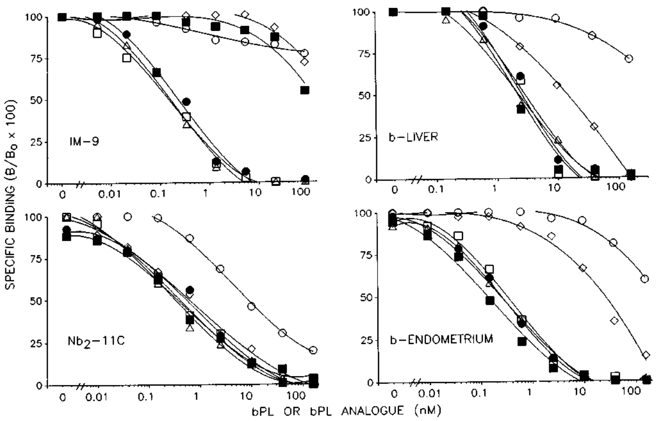

FIG. 2. **Binding of ^{125}I-hGH to homogenates of Nb_2 lymphoma cells or intact IM-9 cells, binding of ^{125}I-bGH to bovine liver microsomal fraction, and binding of ^{125}I-bPL to bovine endometrial microsomal fraction.** Competitive binding was determined by simultaneous addition of bPL (●), bPL(S184H) (■), bPL(S187A) (□), bPL(S187F) (△), bPL(T188F) (◇), and bPL(T188F,I190F) (○). Nonspecific binding was obtained by the addition of 2000 ng/tube hGH or bGH or bPL, respectively. The actual specific binding was 31, 5.5, 5, and 7%, respectively.

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 rbPRLR-ECD (Fig. 5, *B* and *C*) but formed only a 1:1 complex with rPRL-ECD (Fig. 4, *B* and *C*).

We have recently shown by gel filtration experiments that at 1.6 μM 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 μM 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 ^{125}I-bPL binds very poorly in this system, and the K_d of the bPL-bPRLR complex could not be calculated from homologous binding experiments, its affinity toward bPRLR-ECD was evaluated from the IC_{50} values using ^{125}I-hGH as a ligand. The K_d of the hGH:bPRLR-ECD calculated from the data in Fig. 1 was 2.7 nM in agreement to the previously reported value of 2.07 nM (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.

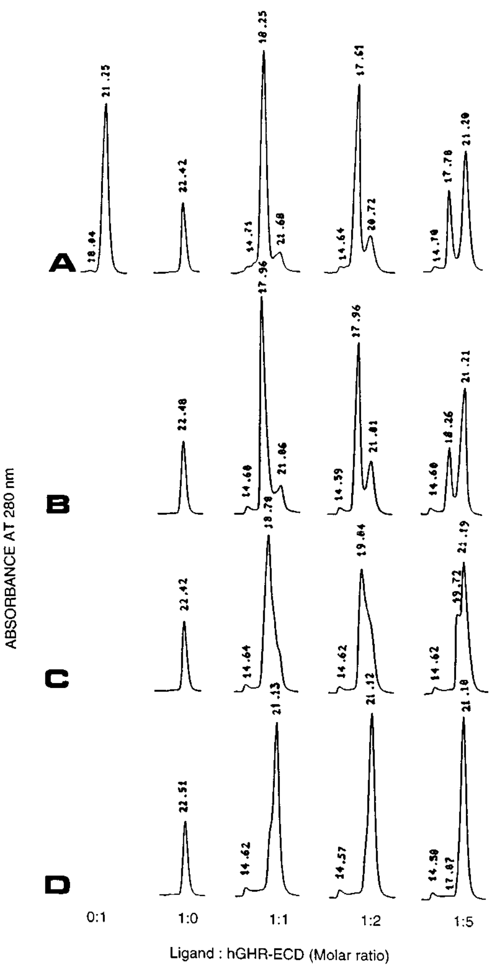

FIG. 3. **Gel filtration chromatography of hGHR-ECD and its complexes with bPL (*A*), bPL(S184H) (*B*), bPL(T188F) (*C*), and bPL(T188F,I190F) (*D*) on a Superdex™ 75 HR 10/30 column.** Protein complexes were preincubated in 1:1, 1:2, and 1:5 bPL or bPL analogue:hGHR-ECD molar ratios. The concentration of hGHR-ECD was constant (15 μM) and the concentrations of bPL or bPL analogues varied from 3 to 15 μM. Protein mixtures were incubated for 15 min at room temperature in TNM buffer and then 200-μl aliquots were applied to the column. The column was developed at 0.5 ml/min. *Ordinate*, absorbance at 280 nm; *abscissa*, time of elution in minutes. The retention times of bovine serum albumin (67 kDa), hGHR-ECD (28 kDa), and bPL (23 kDa) were 16.98, 21.25, and 22.42 min, respectively. For further details, see text.

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 α-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

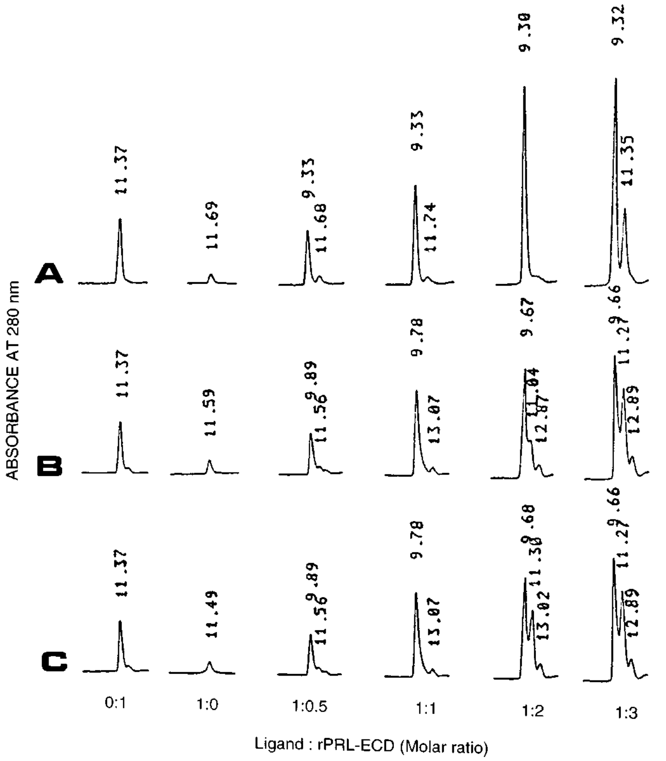

FIG. 4. **Gel filtration chromatography of rPRLR-ECD and its complexes with bPL (*A*), bPL(T188F) (*B*), and bPL(T188F,I190F) (*C*) on a Superdex™ 75 HR 10/30 column.** Protein complexes were preincubated in 1:0.5, 1:1, 1:2, and 1:3 bPL or bPL analogue:rPRLR-ECD molar ratios, using a constant (1.2–1.3 μM) concentration of bPL or bPL analogue and increasing the concentration of rPRLR-ECD. The column was developed at 1 ml/min. The retention times of bovine serum albumin (67 kDa), rPRLR-ECD (25.6 kDa), and bPL (23 kDa) were 9.41, 11.37, and 11.69 min, respectively. For other details see the legend to Fig. 3.

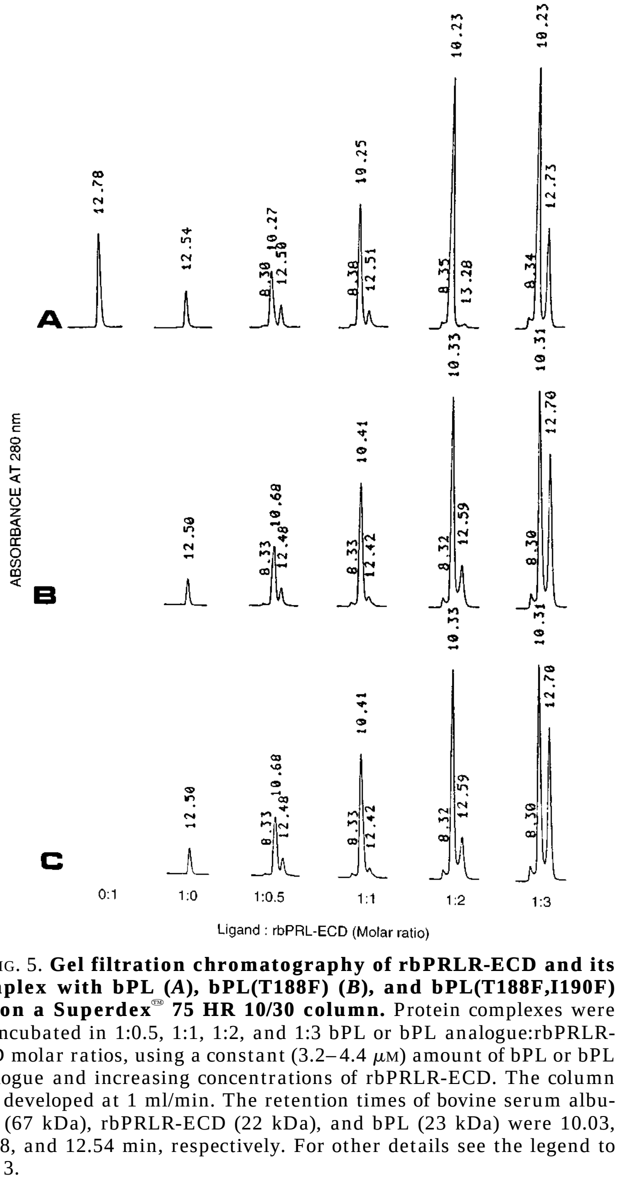

FIG. 5. **Gel filtration chromatography of rbPRLR-ECD and its complex with bPL (*A*), bPL(T188F) (*B*), and bPL(T188F,I190F) (*C*) on a Superdex™ 75 HR 10/30 column.** Protein complexes were preincubated in 1:0.5, 1:1, 1:2, and 1:3 bPL or bPL analogue:rbPRLR-ECD molar ratios, using a constant (3.2–4.4 μM) amount of bPL or bPL analogue and increasing concentrations of rbPRLR-ECD. The column was developed at 1 ml/min. The retention times of bovine serum albumin (67 kDa), rbPRLR-ECD (22 kDa), and bPL (23 kDa) were 10.03, 12.78, and 12.54 min, respectively. For other details see the legend to Fig. 3.

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, rat, and bPRLR-ECD) or was only slightly 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 bioactivity 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

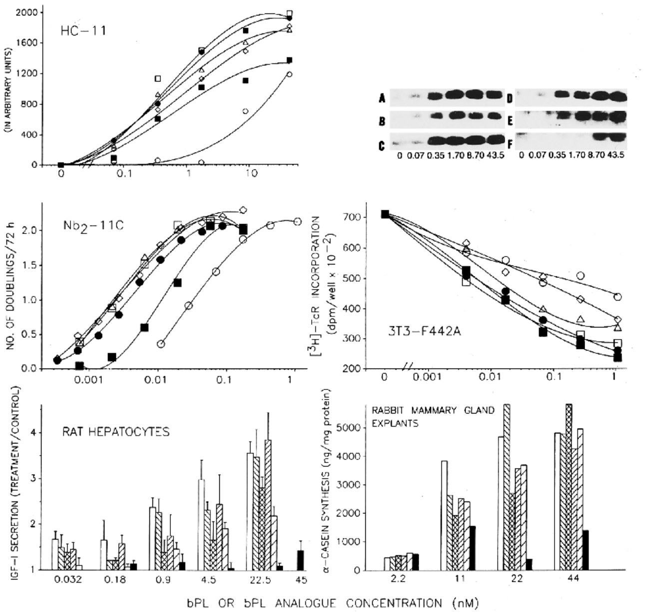

FIG. 6. **Biological activity of bPL and its analogues in five *in vitro* bioassays: β-casein synthesis in HC11 cells, Nb_2-11C lymphoma cell proliferation, antimitogenic somatogenic receptor-mediated activity in 3T3-F442A preadipocytes as shown by curves.** bPL (●), bPL(S184H) (■), bPL(S187A) (□), bPL(S187F) (△), bPL(T188F) (◇), and bPL(T188F,I190F) (○). 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,I190F) (■). The original blot of β-casein synthesis is shown at the *upper right*: bPL (*A*), bPL(S184H) (*B*), bPL(S187A) (*C*), bPL(S187F) (*D*), bPL(T188F) (*E*), and bPL(T188F,I190F) (*F*).

TABLE III

Sequence alignment of hGH, bGH, and bPL (residues 171–191, hGH numbering)

hGH	171	DKVETFLRIV	QCRS.VEGSCG	F	191
bGH	171	HKTETYLRVM	KCRRFGEASCA	F	192
bPL	184	SKISTYINLL	KCR . . . FTPC		200

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 complex to concentrations close to its K_d values obviously leads to dissociation, results of our previous dimerization experiments should be interpreted with caution (24, 25, 37–40), as should those by others (41, 42) performed with micromolar concentrations of the hormones and ECDs. One should also consider to what extent these results reflect binding that occurs under physiological conditions.

The present results enabled us to compare bPL's analogues binding to membrane-embedded receptors in IM-9 human lymphocytes and Nb_2 cells to the corresponding recombinant soluble R-ECDs. In IM-9 cells and hGHR-ECD the different analogues yielded almost identical results, but bPL(T188F) exhibited reduced affinity to soluble rPRLR-ECD, and full affinity to membrane-embedded receptors in Nb_2-11C cells. These results imply that in contrast to hGHR-ECD, the shedding of PRLR-ECDs may cause some structural changes that may affect their binding properties as suggested previously (37).

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 $O^{\gamma 1}$ and Asp-171 $O^{\delta 2}$ form intermolecular hydrogen bonds with Arg-43 $N^{\eta 1}$ and Arg-43 $N^{\eta 2}$ of hGHR-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 facts 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 hGHR-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 with either soluble hGHR-ECD or hGHR embedded in intact IM-9 cells. The area of hPRLRs (Lys-17 and Glu-18, hPRLR-ECD numbering) that corresponds to the first contact area of hGHR binding site one (Arg-43 and Glu-44, hGHR-ECD numbering) is located in almost the same region, as recently evidenced by a three-dimensional analysis. However, in contrast to its interaction with hGHR-ECD, Thr-175 of hGH does not participate in the formation of intermolecular hydrogen bonds with these residues of hPRLR-ECD (21). It should be noted that positions 17 and 18 in rat (45), rabbit (46), and bovine (26) are also occupied by Lys and Glu. The side chain of hGH Thr-175 and most likely also that of bPL's Thr-188 residue are buried in the hormone-PRLR interface (21). However, since bPL(T188F) retains its biological activity in Nb_2 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.

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, but almost completely lost its ability to bind to hGHR-ECD or to GHR in intact IM-9 human lymphocytes. Ser-184 in bPL most likely corresponds to Asp-171 in hGH, which is implicated in the binding to hGHR-ECD and hPRLR-ECD through formation of respective hydrogen bonds with Arg-43 $N\eta 2$ of hGHR-ECD (22) or with Tyr-127 $O\eta$ of hPRLR-ECD (21). Such an interaction most likely does not exist or is modified in nonhuman GHs, in which position 171 is occupied by His (23), and between GHR-ECDs of rabbit (47), rat (48, 49), mouse (50), cow (51), and chicken (52) in which position 43 is occupied by Leu. Therefore, position Asp-171 in hGH and Arg-43 in hGHR seem to be highly important for acquiring species specificity. Although Asp-171 $0^{\delta 2}$ of hGH forms intermolecular hydrogen bonds with Arg-43 $N^{\eta 2}$ of hGHR, its apparent contribution is much less than that of hGH's Thr-175 (44), thus giving potential explanation as to why bPL exhibits binding specificity toward hGHR, despite the Ser-184. The fact that hGH(D171A) retains almost full ability to interact with hGHR-ECD (20) supports this assumption. In contrast, mutation to a larger side chain residue, such as in bPL(S184H) or His-171 in nonhuman GHs, not only prevents formation of the hydrogen bond (which anyhow does not play major role in interaction with the receptor) but also most likely interferes with the interaction of the alkyl portion of hGH's Thr-175 and Lys-172 with Trp-104 of hGHR which is critical to complex formation (22, 44).

Human GH(D171A) has been reported to have a 7.1-fold lower affinity toward hPRLR-ECD (20), most likely because of disrupted interaction with Tyr-127 (hGHR-ECD numbering) or Tyr-98 (hPRLR-ECD numbering), which is one of nine intermolecular hydrogen bonds that stabilize the hGH-hPRLR-ECD interaction (21). Position Tyr-98 (hPRLR-ECD numbering), is also occupied by Tyr in rat (45), rabbit (46), and bovine (53)

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_2 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

1. Murthy, G. S., Schellenberg, C., and Friesen, H. G. (1982) *Endocrinology* **111,** 2117–2124
2. Byatt, J. C., Shimomura, K., Duello, T. M., and Bremel, R. D. (1986) *Endocrinology* **119,** 1343–1350
3. Krivi, G. G., Hauser, S. D., Stafford, J. M., Collier, R. J., and Byatt, J. C. (1989) *Proceedings of the 71st Meeting of the Endocrine Society, June 21–24, 1989, Seattle, Washington*, Abstr. 1523, Endocrine Society, Bethesda, MD
4. Byatt, J. C., Welply, J. K., Leimgruber, R. M., and Collier, R. J. (1990) *Endocrinology* **127,** 1041–1049
5. Glenn, K. C., Rose, K. S., and Krivi, G. G. (1988) *J. Cell. Biochem.* **37,** 371–383
6. Vashdi, D., Elberg, G., Sakal, E., and Gertler, A. (1992) *FEBS Lett.* **305,** 101–104
7. Shamay, A. (1989) *Control of Proliferation of Bovine Mammary Epithelial Cells in Vitro by Hormones and Growth Factors.* Ph.D Thesis, The Hebrew University of Jerusalem, Israel
8. Galosy, S. S., Gertler, A., Elberg, G., and Laird, D. M. (1991) *Mol. Cell. Endocrinol.* **78,** 229–236
9. Gertler, A., Hauser, S. D., Sakal, E., Vashdi, D., Staten, N., Freeman, G. G., and Krivi, G. G. (1992) *J. Biol. Chem.* **267,** 12655–12659
10. Vashdi-Elberg, D., Staten, N. R., Sakal, E., Krivi, G. G., and Gertler, A. (1995) *Endocrinology* **136,** 1258–1266
11. Abdel-Meguid, S. S., Shieh, H. S., Smith, W. W., Dayringer, H. E., Violand, B. M., and Bentle, L. A. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84,** 6434–6437
12. Gertler, A., Shamay, A., Cohen, N., Ashkenazi, A., Friesen, H. G., Levanon, A., Gorecki, M., Aviv, H., Hadari, D., and Vogel, T. (1986) *Endocrinology* **118,** 720–726
13. Ashkenazi, A., Vogel, T., Barash, I., Hadary, D., Levanon, A., Gorecki, M., and Gertler, A. (1987) *Endocrinology* **121,** 414–419
14. Binder, L., Vogel, T., Hadary, D., Elberg, G., and Gertler, A. (1989) *Mol. Endocrinol.* **3,** 923–930
15. Binder, L., Gertler, A., Elberg, G., Guy, R., and Vogel, T. (1990) *Mol. Endocrinol.* **4,** 1060–1068
16. Tchelet, A., Gertler, A., Sakal, E., Djiane, J., and Vogel, T. (1994) *Hormone Res.* **41,** 103–112
17. Towns, R., Kostyo, J. L., Vogel, T., Sakal, E., Tchelet, A., Maher, R., and Gertler, A. (1992) *Endocrinology* **130,** 1225–1230
18. Cunningham, B. C., and Wells, J. A. (1989) *Science* **244,** 1081–1086
19. Cunningham, B. C., Shurami, P., Ng, P., and Wells, J. A. (1989) *Science* **243,** 1330–1336
20. Cunningham, B. C., and Wells, J. A. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 3407–3411
21. Somers, W., Ultsch, M., De Vos, A. M., and Kossiakoff, A. A. (1994) *Nature* **372,** 478–481
22. De Vos, A., Ultsch, M., and Kossiakoff, A. A. (1992) *Science* **255,** 306–312
23. Nicoll, C. S., Mayer, G. L., and Russell, S. M. (1986) *Endocr. Rev.* **7,** 169–203
24. Bignon, C., Sakal, E., Belair, L., Chapnik-Cohen, N., Djiane, J., and Gertler, A. (1994) *J. Biol. Chem.* **269,** 3318–3324
25. Tchelet, A., Sakal, E., Vogel, T., Krivi, G. G., Creely, D., Reichman, A., and Gertler, A. (1993) *Adolesc. Pediatr. Endocrinol.* **24,** 114–126
26. Tchelet, A., Staten, N. R., Creely, D. P., Krivi, G. G., and Gertler, A. (1995) *J. Endocrinol.* **144,** 393–403
27. Sandowski, Y., Nagano, M., Bignon, C., Djiane, J., Kelly, P. A., and Gertler, A. (1995) *Mol. Cell. Endocrinol.* **115,** 1–11
28. Obukowicz, M. G., Staten, N. R., and Krivi, G. G. (1992) *Appl. Environ. Microbiol.* **58,** 1511–1523
29. Laemmli, U. K. (1970) *Nature* **227,** 680–685
30. Yang, J. T., Wu, C-S. C., and Martinez, H. M. (1986) *Methods Enzymol.* **130,** 208–269
31. Chen, Y. H., Yang, J. T., and Martinez, H. M. (1972) *Biochemistry* **11,** 4120–4131
32. Gill, S. C., and von Hippel, P. H. (1989) *Anal. Biochem.* **182,** 319–326
33. Gertler, A., Walker, A., and Friesen, H. G. (1985) *Endocrinology* **116,** 1636–1644
34. Jahn, G., Dusanter-Fourt, I., Kelly, P. A., Houdebine, L.-M., and Djiane, J. (1987) *Proc. Soc. Exp. Biol. Med.* **184,** 19–23
35. Deleted in proof
36. Deleted in proof
37. Gertler, A., Bignon, C., Staten, M. R., Sakal, E., Tchelet, A., Krivi, G. G., and Djiane, J. (1994) *Proc. Soc. Exp. Biol. Med.* **206,** 273–279
38. Sakal, E., Tchelet, A., Uchida, E., Shimokawa, S., Nishikawa, S., Hayakawa, T., Krivi, G. G., and Gertler, A. (1993) *Mol. Cell. Endocrinol.* **65,** 145–155
39. Gertler, A., Petridou, B., Krivi, G. G., and Djiane, J. (1993) *FEBS Lett.* **319,** 277–281
40. Staten, N. R., Byatt, J. C., and Krivi, G. G. (1993) *J. Biol. Chem.* **268,** 18467–18473
41. Cunningham, B. C., Ultsch, M., Kossiakoff, A. A., De Vos, A., Mulkerrin, M. G., Clauser, K. R., and Wells, J. A. (1991) *Science* **254,** 821–825
42. Hooper, K. P., Padmanabhan, R., and Ebner, K. E. (1993) *J. Biol. Chem.* **268,** 22347–22352
43. Lowman, H. B., and Wells, J. A. (1993) *J. Mol. Biol.* **234,** 564–578
44. Clackson, T., and Wells, J. A. (1995) *Science* **267,** 383–386
45. Boutin, J. M., Jolicoeur, C., Okamura, H., Gagnon, J., Edery, M., Shirota, M., Banville, D., Dusanter-Fort, I., Djiane, J., and Kelly, P. A. (1988) *Cell* **53,** 69–73
46. Edery, M., Jolicoeur, C., Levi-Meyrueis, C., Dusanter-Fourt, I., Petridou, B., Boutin, J. M., Lesuer, L., Kelly, P. A., and Djiane, J. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86,** 2112–2116
47. Leung, D. W., Spencer, S. A., Cachianes, G., Hammonds, R. G., Collins, C., Henzel, W. H. J., Barnard, R., Waters, M. J., and Wood, W. I. (1987) *Nature* **330,** 537–543
48. Baumbach, W. R., Horner, D. I., and Logan, J. S. (1989) *Genes & Dev.* **3,** 1199–1205
49. Mathews, L. S., Enberg, B., and Norstedt, G. (1989) *J. Biol. Chem.* **264,** 9905–9910
50. Smith, W. C., Kuniyoshi, J., and Talamantes, F. (1989) *Mol. Endocrinol.* **3,** 984–990
51. Hauser, S. D., McGrath, M. F., Collier, R. J., and Krivi, G. G. (1990) *Mol. Cell. Endocrinol.* **72,** 187–200
52. Burnside, J., Shuenn, S. L., and Cogburn, A. (1991) *Endocrinology* **128,** 3138–3192
53. Scott, P., Kessler, M. A., and Schuler, L. (1992) *Mol. Cell. Endocrinol.* **89,** 47–58