Novel Recombinant Analogues of Bovine Placental Lactogen

G133K AND G133R PROVIDE A TOOL TO UNDERSTAND THE DIFFERENCE BETWEEN THE ACTION OF PROLACTIN AND GROWTH HORMONE RECEPTORS

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Placental lactogens (PLs)1 are 22–23-kDa proteins, secreted by placentas of primates, rodents, and ruminants (1). These proteins are structurally related to growth hormone (GH) and prolactin (PRL) and probably share the same mechanism of action. However, bPL and oPL (2) are unusual in that they are capable of recognizing and subsequently exhibiting their biological activity through both lactogenic and somatogenic receptors. It has been shown by us (3) and others that bPL is indeed capable of acting through PRLRs in bovine mammary glands (4), in the rat Nb2 lymphoma cell line (5), and through somatogenic receptors in 3T3-F442A preadipocytes (6) and rat hepatocytes (7). We have also shown that these two activities may be selectively modified. For instance, in bPL(T188F) or bPL(K73D/F/A), the binding to full-size somatogenic receptors or their ECDs and to bPLR in the endometrium, as well as somatogenic receptor-mediated biological activities, were reduced or abolished, whereas binding to lactogenic receptors or their ECDs and subsequent biological activity was either fully or almost fully retained (7, 8). More recently, we have mutated Lys73, which corresponds to Arg84 in hGH and has been identified to be important in the interaction with hGHR-ECD but not with hPRLR-ECD (9). Results of that study suggested that position 73 in bPL is more important for somatogenic than lactogenic properties and indicated that the difference could be related to the possibility that the minimal duration of the existence of the hormone-induced receptor dimer required for the initiation of the biological signal by lactogenic or somatogenic receptors may be different (8). In the present study we mutated Gly133, which probably corresponds to Gly133 and Gly120 in bGH and hGH. In these hormones, replacement of Gly by a large, positively charged side chain interfered with the formation of a homodimeric complex and led to the appearance of antagonistic activity (10, 11). We could therefore expect that analogous mutation in bPL, namely G133K and G133R, would lead to a similar change. Binding of site 2 of the bPL to both lactogenic and somatogenic receptors and its subsequent ability to homodimerize the extracellular domain of GH or PRL receptors were reduced to a similar degree. Surprisingly however, only the somatogenic receptor-mediated biological activity was abolished, whereas the activity mediated through lactogenic receptors was almost fully retained.

Two new analogues of bovine placental lactogen (bPL), bPL(G133K) and bPL(G133R), were expressed in Escherichia coli, refolded, and purified to a native form. Binding experiments, which are likely to represent the binding to site 1 only, to intact FDC-P1 cells transfected with rabbit (rb) growth hormone receptor (GHR) or with human (h) GHR, to Nb2 rat lymphoma cells, or to rabbit mammary gland membranes prolactin receptor (PRLR), revealed only small or no reduction in binding capacity. The complex formation between these analogues and receptor extracellular domains (R-ECD) of various hormones was determined by gel filtration. Wild type bPL yielded 1:2 complex with hGHR-ECD, rat PRLR-ECD, and rbPRLR-ECD, whereas both analogues formed only 1:1 complexes with all R-ECDs tested. Real time kinetics experiments demonstrated that the ability of the analogues to form homodimeric complexes was compromised in both PRLR- and GHR-ECDs. The biological activity transduced through lactogenic receptors in in vitro bioassays in rabbit mammary gland acini culture and in Nb2 cells was almost fully retained, whereas the activity transduced through somatogenic receptors in FDC-P1 cells transfected with rbGHRs or with hGHRs was abolished. Both analogues exhibited antagonistic activity in the latter cells. To explain the discrepancy between the effect of the mutation on the signal transduced by PLR versus GHRs we suggest that: 1) the mutation impairs the ability of site 2 of bPL to form a stable homodimeric complex with both lactogenic and somatogenic receptors by a drastic shortening of the half-life of 2:1 complex; 2) the transient existence of the homodimeric complex is still sufficient to initiate the signal transduced through lactogenic receptors but not through somatogenic receptors; and 3) one possible reason for this difference is that JAK2, which serves as a mediator of both receptors, is already associated with lactogenic receptors prior to hormone binding-induced receptor dimerization, whereas in somatogenic receptors the JAK2 receptor association occurs subsequently to receptor dimerization.

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The abbreviations used are: PL, placental lactogen; PRL, prolactin; PRLR, PRL receptor; GH, growth hormone; GHR, GH receptor; ECD, extracellular domain; R-ECD, receptor ECD; b, human; b, bovine; o, ovine; r, rat; rb, rabbit; RU, resonance unit; PAGE, polyacrylamide gel electrophoresis; SPR, sulfon plasmon resonance; HBS, Hepes-buffered saline; PCR, polymerase chain reaction; RT, retention time.
EXPERIMENTAL PROCEDURES

Materials — Recombinant hGH was obtained from Biotechnology General Inc. (Rehovot, Israel). Recombinant bPL, nonglycosylated rabbit (tb) and rat (r) PRLR-ECDs and hGHR-ECD were prepared as described previously (12–15). Carrier-free Na[125I] was purchased from New England Nuclear Corp. (Boston, MA). Molecular weight markers for gel electrophoresis, RPMI 1640 medium, lysozyme, naldixic acid, Triton X-100 (detergent), bovine serum albumin (BIA grade) were obtained from Sigma. SDS-PAGE reagents and a Protein Assay kit were purchased from Bio-Rad. Fetal calf serum and horse serum were purchased from Labotal Co. (Jerusalem, Israel), and a Superdex 75 HR 10/30 column and Q-Sepharose (fast flow) were obtained from Amersham Pharmacia Biotech. Reagents for sputum plasminogen resonance (SPR) including CM5 sensor chips, Hepes-buffered saline (HBS), N-hydroxysuccinimide, N-ethyl-(N,N-diethylaminopropyl) carbodiimide, 2-mercaptoethanol, ethanolamine hydrochloride, and ethylene glycol were obtained from Amersham Pharmacia Biotech. All other chemicals were of analytical grade.

Construction of bPL Analogue Expression Vectors—Synthetic gene fragments for each bPL analogue were constructed using polymerase chain reaction (PCR) technology. PCR fragments containing the desired mutations and appropriate restriction enzyme sites were initially generated using synthetic oligonucleotides (primers) and pMON3922 DNA template (16). The forward mutant primers contained the desired mutations at amino acid 133 and encoded an XhoI restriction enzyme site: (AAAGTACTTCTAGAAGTGGAAATGATACAAA for G133K), (AAAGTACTTCTAGAACGTGTGGAAATGATACAAA for G133R and (AAAGTACTTCTAGAACGTGTGGAAATGATACAAA for G133K), whereas the reverse primer was complementary to the immediate immediate NcoI gene and the XhoI site was located in pMON3922. The PCR products (~200 base pairs), which encode the C-terminal portion of the bPL gene with the mutations, were purified using a Wizard PCR kit (Promega, Madison, WI) and digested with XhoI and HindIII restriction enzymes. For each bPL analogue, a three-way ligation was set up consisting of the PCR fragment, the NcoI-XhoI N-terminal bPL gene fragment from pMON2922 (~400 base pairs), and the HindIII-NotI vector pMON3401 (17). The constructs were then transformed into MAX Efficiency DH10B competent cells, and colonies were grown up in LB medium containing 75 μg/ml spectinomycin at 37 °C overnight. Plasmid DNA was isolated using a Plasmid Midi kit (Qiagen, Chatsworth, CA), sequenced with AmpliTaq FS and dye terminators, and analyzed on an ABI 373 Sequencer. Plasmid DNA from each of the bPL analogues with the correct mutation was used to transform MON105 cells (17) for expression in the cytoplasm as insoluble aggregates.

Expression, Refolding, and Purification of bPL Analogues—Escherichia coli MON105 cells transformed with the expression plasmids containing the bPL variant genes were incubated in 500 ml of Terrific Broth medium (18) by shaking at 200 rpm at 37 °C in 2-liter flasks to an optical density of 0.5 and harvested by 5-min centrifugation at 10,000 × g. The pellets were then injected at a concentration of 100 μg/ml in 10 ml sodium acetate buffer, pH 5.4, yielding 1000–2000 resonance units (RU) of immobilized hormone. Nonreacted sites were blocked with an 8-min injection of 1 m ethanolamine hydrochloride at pH 8.5. Binding capacities were checked by repeated injections of 5 μl R-ECDs in HBS. Immobilized bPL or its analogues could be regenerated over 50 runs with 4.5 mg of MgCl2 pulses (1–2 min).

Kinetic Measurements of R-ECD-Hormone Interactions — All experiments were performed at a flow rate of 5 μl/min in HBS at 25 °C. Once the hormone being tested was covalently immobilized among amino group couplings, serial dilutions of each R-ECD were injected for 480 s (70–550 m on the scale in Fig. 4) and then washed with HBS for 720 s (550–1270 s) prior to regeneration. Because the recombinant R-ECDs had been lyophilized with sodium bicarbonate buffers at a salt-protein ratio of 1:2, bulk refractive indexes varied with sample dilution, and these variations were corrected by injecting the same dilutions into flow cells in which unrelated ligands had been immobilized.

Analysis and Calculation of Kinetic Constants—BLAcore incorporated software (BIA Evaluation and BIA Simulation, version 2.1) allowed us to fit experimental curves with 1:1 or 1:2 association/disassociation models and calculate the probabilities of each being the most accurate representation of reality (26) and to calculate kinetic constants with standard deviations. Reverse verification of calculated data was performed by simulating the interaction assuming a variable relative occupation of the two sites.

In Vitro Bioassays — Two in vitro bioassays in which the signal was transduced through lactogenic receptors were performed: rat Nb2–11C lymphoma-cell-proliferation bioassay, in which the original protocol was slightly modified (27), and β-casein production in rabbit mammary gland acini. Two in vitro bioassays in which the signal was transduced through somatogenic receptors were based on the proliferation of FDC-P1 cells transfected with rabbit (clone FDC-P1-3B9) or human (clone FDC-P1-9D11) GHRs (21, 22). Cells cultured in RPMI 1640 medium supplemented with 5% fetal calf serum and hGH (100 ng/ml) were washed in phosphate-buffered saline, resuspended in RPMI 1640 medium supplemented with 5% horse serum at a concentration of 50,000 cells/ml, and plated (1 ml/well) in 24-well plates. Various concentrations of bPL and/or its analogues were then added, and the cells were grown for an additional period of 72 h. Cell growth was determined by counting the cells with a Coulter counter (Coulter Electronics Inc, Hialeah, FL), and the number of doublings was calculated as described previously (27).

RESULTS

Expression of bPL Analogues in E. coli — One clone of each E. coli bacteria carrying the mutant plasmids was grown in Terrific Broth medium, and the protein expression was induced by...

2. N. Daniel and J. Djiane, unpublished data.
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**FIG. 1.** SDS-PAGE analysis of the expression of bPL analogues. MON 105 E. coli bacteria strain transformed with pMON3401 plasmid carrying bPL analogues DNA. bPL(G133K) (lanes 2 and 3) and bPL(G133R) (lanes 4 and 5) clones were grown in Terrific Broth medium induced with (lanes 2 and 5) or without (lanes 3 and 4) naldixic acid. Recombinant bPL used as a marker (lanes 1 and 6) is marked with arrowheads.

naldixic acid. SDS-PAGE of the bacterial pellet in the presence of β-mercaptoethanol revealed a conspicuous band of a molecular weight the same as that of the recombinant bPL that was used as a marker (Fig. 1).

**Purification of bPL Analogues**—The profile of bPL analogues elution from a Q-Sepharose column shows that over 60% of the protein was eluted with 0.15 M NaCl (not shown). Every third tube was analyzed for monomer content, and fractions containing >98% pure monomer were pooled, dialyzed against NaHCO₃ (1:5 salt:protein ratio), and lyophilized. This fraction was further used for binding and biological studies. Fractions eluted with 0.4 M NaCl consisted mainly of oligomers (not shown). SDS-PAGE of the pooled monomer fraction, performed with and without β-mercaptoethanol, revealed only one band with a molecular mass of 23 kDa (not shown). The oligomeric fraction eluted at 0.4 M NaCl also yielded mostly a 23-kDa band, indicating that the oligomers were formed by noncovalent interactions. Analysis of the α-helix content (Table I) revealed values close to (and within experimental error) those of bPL, indicating proper refolding.

**Binding Experiments**—The ability of analogues to compete with [125I]hGH and bind somatogenic or lactogenic receptors was analyzed in three intact cell lines and compared with wild type bPL. bPL(G133K) was found to be 60% more potent than bPL, and bPL(G133R) was found to be just as potent as bPL in binding to the mouse myeloid cell line FDC-P1 transfected with somatogenic rbGH receptor (Fig. 2A). The results with the same cell line transfected with hGH receptor revealed that bPL(G133K) did not lose any of its binding ability, and bPL(G133R) retained 50% of its binding capacity as compared with wild type bPL (Fig. 2B). A slightly larger loss in the binding capacity was observed in both analogues when the Nb2-11C cell line, carrying the lactogenic receptors, was used. In those cells, bPL(G133K) and bPL(G133R) lost 75 and 80% of their binding ability, respectively (Fig. 2C). In contrast, the binding capacity of both analogues to lactogenic receptor in the microsomal fraction of rabbit mammary gland was hardly affected (Fig. 2D). In all cases, the results of the binding experiments gave better fit to a one-site than a two-site model.

**Gel Filtration Experiments**—The stoichiometry of the complexes formed by interaction of R-ECDs of somatogenic or lactogenic and bPL analogues was determined by gel filtration at several R-ECD:analogue ratios, at a constant concentration of the latter (2 μM). Measuring the size of the eluted peaks and their retention times enabled us to analyze and calculate the stoichiometry and the molecular weight of the complex formed. Whereas bPL was capable of dimerization of hGH-R-ECD, both analogues formed only a 1:1 complex even in the highest concentration of R-ECD (Fig. 3A). Similar results were obtained by measuring the complexes formed with rPRLR-ECD and rbPRLR-ECD (Fig. 3, B and C).

**TABLE I**

| Analogue          | α-Helix | β-Sheet | β-Turn | Random coil |
|-------------------|---------|---------|--------|-------------|
| bPL wild type     | 52      | 5       | 9      | 34          |
| bPL(G133K)       | 47.3 ± 4.1 | 0.0 ± 24.0 | 18.9 ± 7.9 | 33.8 ± 4.1 |
| bPL(G133R)       | 45.7 ± 4.2 | 0.0 ± 24.4 | 19.2 ± 8.1 | 35.1 ± 4.2 |

*Ref. 8.

**SPR Determination of the Interaction between hGHR-ECD, rPRLR-ECDs, and rbPRLR-ECDs and bPL, bPL(G133K), and bPL(G133R)**—The interactions of bPL with hGHR-ECD, rPRLR-ECD, and rbPRLR-ECD (Fig. 4, upper panels) were analyzed by comparison with a theoretical model using chi-square analysis. In all three cases the interactions proved to be unsuited to the 1:1 model and strongly favored the two-site model (Table II), thus confirming our previous results (26). Both mutations resulted in a decrease of the apparent stoichiometry. However, whereas in the interactions with hGHR-ECD and rbPRLR-ECD the decrease was moderate (ECD:hormone molar ratio of 0.6–1.0), resulting most likely from the increased kₐ values of site 2, a drastic decrease was observed in apparent stoichiometry with rPRLR-ECD (Table II and Fig. 4, middle and lower panels). In the latter case the results clearly show that the kₐ values for both sites 1 and 2 were affected, and a very low RU signal (5–10% compared with the wild type hormone) at the steady state even at the 1000 nM concentrations of the rPRLR-ECD was observed. To facilitate the comparison, the top lines in these figures represent the interaction with the wild type bPL (1000 nM) copied from the upper panel, and the same scale was used for each ECD.

Analysis of the data presented in Fig. 4 (Table II) revealed that the kₐ constants of both analogues for both site 1 and site 2 were changed only slightly (the interaction with rbPRLR-ECD) or moderately (the interaction with hGHR-ECD). The interaction of the analogues with the rPRLR-ECD resulted in a very low RU signal that did not allow us to calculate the kₐ constants. The main effect of both mutations was an increase of the kₐ for site 2. This increase was high (−15–20-fold) in the case of rbPRLR-ECD, which also explains the over 2–3-fold decrease in the apparent stoichiometry (Fig. 4, right panels) and lower (1.5–2-fold) in the case of hGHR-ECD (Fig. 4, middle panels). In the case of rPRLR-ECD the increase was 30–35-fold. However, in the latter case the mutations have also caused a 20–30-fold increase in the kₐ off site 1 leading to drastic reduction in the amount of the complex observed at the steady state (Fig. 4, middle panels). Kinetic analysis of the association phase and the dissociation phase (as determined respectively 200–300 and 580–650 s on the time scale in Fig. 4) revealed very good fit to the two-site model and a poor fit to the one-site model. Analysis of the dissociation rate (750–800 s on the time scale in Fig. 4) revealed very good fit to the two-site model, however, showed better match to the 1:1 model than to 2:1 model, most likely because of the short time scale in Fig. 4), however, showed better match to the 1:1 model than to 2:1 model, most likely because of the short half-life of the 2:1 complex. All together the mutations resulted a −10-fold increase in Kₐ for site 2 for hGHR-ECD and corresponding 32–56- and 50-fold increases for rat and rbPRLR-ECDs. As mentioned above in the case of rPRLR-ECD, the Kₐ for site 1 was also elevated by 20–28-fold. The calculations of both Kₐ values for rPRLR-ECD provide, however, an approximate value only because the kₐ constants used for calculation were those of the wild type hormone. In conclusion, the SPR data show that both mutations destabilized the 2:1 complex in all three cases and also the 1:1 complex in the case of rPRLR-ECD. The decrease in the stability of 1:1 complex of both analogues with rPRLR-ECD is likely the main reason for the partial loss of activity in the Nb₂ bioassay (see below).
**DISCUSSION**

The CD spectra of both bPL(G133K) and bPL(G133R) were almost identical to that of wild type hormone, indicating that mutations at position 133 did not change the overall secondary structure and that the analogues were refolded correctly. This conclusion was further verified by the finding that the in vitro biological activity of both analogues mediated by heterologous lactogenic receptors was fully retained in rabbit mammary gland acini culture and only partially reduced in Nb2-11C rat lymphoma cells. Therefore the changes resulting from the G133K or G133R mutation should be attributed to a specific local effect rather than to an incorrect refolding.

Binding of the bPL and bPL analogues to somatogenic and lactogenic receptors was studied using three independent methods: gel filtration of their complexes with soluble recombinant extracellular domains of hGHR and rat and rabbit PRLRs, binding experiments to intact cells with somatogenic or lactogenic receptors or to microsomal fraction from rabbit mammary gland, and SPR methodology, which was already proven to be an excellent tool for studying the real time kinetics of binding to several soluble lactogenic and somatogenic receptors (26). Gel filtration experiments indicated that unlike the wild type bPL both bPL(G133R) and bPL(G133K) lost their ability to homodimerize soluble ECDs of both rat and rabbit PRLRs and hGHR. In contrast to these findings, binding experiments of both analogues to hGH and rbGH receptors in intact cells indicated no change or only slight change in comparison with bPL. Binding to the lactogenic receptors was only partially reduced for both analogues. The discrepancy obtained by the two methods may be explained by an assumption that the binding experiments of both wild type hormone and both analogues show binding to site 1 only, whereas in gel filtration experiments the wild type hormone is capable of forming a 1:2 complex. This conclusion is further substantiated by the facts that: 1) in all cases the results of binding experiments gave a better match to the one-site than the two-site model and 2) the gel filtration experiments were performed at 2 mM complex concentration, in contrast to the binding assays, carried out at pm to nm concentrations. Thus, in the case of bPL the high concentrations of the reagents favor the appearance of the 2:1 complex in gel filtration but not in the binding experiments.

A detailed kinetic analysis by SPR was performed because neither the gel filtration nor the binding experiments provided a sufficient understanding of all the changes in binding properties. The advantage of the kinetic analysis stems from its ability to deduce the stoichiometry of interaction, even in very transient ligand-receptor interactions, and to permit calculations of the kinetic constants. Kinetic analysis and simulation revealed that in all cases both analogues were capable of forming a 1:2 complex with the respective R-ECDs the mutations destabilized the complex mainly because of an increase in $k_{off}$ resulting in a more efficient off-rate rather than to an incorrect refolding.

**Biological Activity in Vitro—**Proliferation experiments with mouse myeloidic FDC-P1 cells transfected with rbGHR (3B9 cell line) or hGHR (9D11 cell line) show that the somatogenic-receptor-mediated biological activity of both analogues was completely abolished (Fig. 5, A and B). In contrast, both analogues retained substantial biological activity in two bioassays in which the signal was transduced through lactogenic receptors. Proliferation assay of Nb2-11C cells revealed only 3- and 6-fold loss of activity for bPL(G133K) and bPL(G133R), respectively (Fig. 5C). In another bioassay, in which casein secretion in rabbit mammary gland acini was determined, both analogues were as active as wild type bPL (Fig. 5D). To investigate the antagonistic properties of the analogues, the same somatogenic and lactogenic receptor-mediated bioassays were used. In those assays, we measured the ability of the analogues to inhibit proliferation of bPL-stimulated cells in FDC-P1 cells transfected with rbGHR or hGHR and in Nb2-11C cells expressing rPRLR. In both somatogenic FDC-P1 cell lines inhibition was observed when treating the cells at 0.18 nM bPL concentration, which resulted in a submaximal cell proliferation rate. Both bPL(G133K) and bPL(G133R) produced half-maximal inhibition of activity in FDC-P1 cells transfected with rbPRL at concentrations of 0.13 and 0.27 nM, respectively (Fig. 6A). The inhibitory activity was also observed in the FDC-P1 cell line transfected with hGHR, although about 100-fold higher concentrations (12 and 19 nM, respectively) of both bPL(G133K) and bPL(G133R) were required to achieve the same extent of inhibition (Fig. 6B). In contrast, addition of bPL analogues in 10–100-fold excess to Nb2-11C cells stimulated with 0.011 nM bPL (which is the suboptimal concentration) had no inhibitory effect but had an additive effect and elevated the cell growth to maximum (not shown).
ing $K_d$ value. The interaction of both analogues with rPRLR-ECD was much more affected than the interaction with rbPRLR-ECD or hGHR-ECD, likely because of the increase in the $k_{off}$ constants for site 1. This interpretation is supported by the competitive binding experiments (Fig. 2C) and by the fact that the biological activity in the rat lymphoma Nb2 cells was reduced.

The results presented in this paper raise fundamental questions about the connection between the binding of bPL and its analogues to somatogenic or lactogenic receptors and the biological activity transduced as a result of this interaction. It was shown that despite the reduced affinity of site 2 of the analogues toward both somatogenic and lactogenic receptors, dramatic reduction in biological activity and even conversion of the agonist to antagonist was observed in events mediated through somatogenic receptors only. In contrast, in events mediated through lactogenic receptors, in which the effect of mutation on the binding to lactogenic receptors or their ability to homodimerize the PRLR-ECDs was strongly impaired, the lactogenic receptor-mediated biological activity was not, or was only slightly, affected.

Although the three-dimensional structure of bPL is not known, we have recently found that the related hormone oPL has two binding sites and forms a 1:2 complex with rPRLR-ECD (28), suggesting that a similar complex may also be formed with bPL. Structural analysis of the complex suggests that Gly133 of the hormone is located close to the receptor Trp72, which is homologous to Trp 104 in hGHR and is conserved in all GH and PRL receptors (29). Replacement of Gly by a large, positively charged side chain interferes with the formation of a homodimeric complex, and in the case of hGH (11, 30) or bGH (30) this mutation led to the appearance of antagonistic activity. We could therefore expect that a similar mutation in bPL, namely G133K or G133R, would lead to an appearance of antagonistic activity toward lactogenic receptors.

3 A. M. De Vos, personal communication.
as well. As shown above, at least in the two tested models (rabbit mammary gland acini culture and Nb2 lymphoma cells), this did not happen.

One possible explanation could be that dimerization of lactogenic receptors is not obligatory for initiation of transduced signal. Such a suggestion seems extremely unlikely in light of both direct (31) and indirect evidence (26, 32), which shows the opposite, and by the recent crystallographic analysis that shows

![FIG. 4. Association and dissociation kinetics of hGHR-ECD (A), rPRLR-ECD (B), and rbPRLR-ECD (C) with bPL (upper panel), bPL(G133K) (middle panel), and bPL(G133R) (lower panel). The hormones were covalently linked to CM-dextran through amino groups. Resonance signals (RU) were plotted as a function of time for several concentrations (1000, 500, 250, 125, 62, and 31 nM from the top to the bottom) of various R-ECDs. The association phase was carried out through 600 s, after which the infusion of the soluble R-ECDs was stopped and the dissociation phase in continuous buffer flow was monitored for an additional period of 600 s. To facilitate the comparison the upper lines in middle and lower panels represent the interaction with the wild type bPL (1000 nM) copied from the upper panel.](image)

TABLE II
Calculation of kinetic and thermodynamic constants of sites 1 and 2 for the interaction between bPL, bPL(G133R), and bPL(G133K) with rabbit and rat PRLR-ECDs and hGHR-ECD

| Hormone (sites 1 and 2) | Interaction with hGHR-ECD | Interaction with rPRLR-ECD | Interaction with rbPRLR-ECD |
|------------------------|---------------------------|--------------------------|----------------------------|
|                        | $k_{on}$ $(mol^{-1}s^{-1} \times 10^4)$ | $k_{off}$ $(s^{-1} \times 10^{-4})$ | $K_d$ (nm) | ECD:hormone (molar ratio) | Half-life (min) |
| bPL                    | 1 6.3 2.1 3.4 1.4:1 55 | | | | |
| bPL(G133K)             | 1 0.9 2.6 29 0.7:1 44 | | | | |
| bPL(G133R)             | 1 4.2 2.0 4.8 1.0:1 58 | | | | |
| bPL(G133R)             | 2 2.5 166 684 0.7 | | | | |
| bPL                    | 1 3.6 2.0 5.6 1.5:1 58 | | | | |
| bPL(G133K)             | 1 2.1 10 47 | | | |
| bPL(G133R)             | 1 ND 314 | | | |
| bPL(G133R)             | 2 4.0 136 340 0.9 | | | |
| bPL                    | 2 3.6 2.8 7.8 0.9:1 41 | | | |
| bPL(G133K)             | 2 ND 354 | | | |
| bPL(G133R)             | 2 5.1 190 372 0.6 | | | |

*a* Apparent stoichiometry at 1000 ng ECD/ml as determined by surface plasmon resonance from the data presented in Fig 4.

*b* From Helman et al. (8).

*c* ND, not determined because very low RU did not allow accurate calculations.

*d* The $K_d$ values are only approximate because they were calculated not from the corresponding analyses but based on an as yet unproven but reasonable assumption that the mutations did not change the $k_{on}$ constants of the wild type hormone.
formation of an 1:2 oPL-rPRLrECD complex (28). However, an alternative explanation may be proposed in view of our recent suggestion that transient dimerization of PRLRs, lasting a few seconds or less, is sufficient to elicit full biological response (26, 33). This assumption is supported by the finding that after the homodimeric complex is formed, receptor-associated JAK2 or other kinases are instantly activated by mutual transphosphorylation, forming docking sites for other downstream proteins (34, 35). Once this occurs, the receptor dimers are no longer needed. This hypothesis is likely to be true for biological events induced through both lactogenic and somatogenic receptors. The present experiments indicate, however, that the minimal duration of the existence of the homodimeric complex required for the initiation of the biological signal may be shorter for the lactogenic than for the somatogenic receptors. A possible reason for this difference is the recent finding that JAK2, which serves as a mediator of both receptors, is already associated with lactogenic receptors prior to hormone binding-induced receptor dimerization, whereas in somatogenic receptors the JAK2-receptor association occurs subsequently to receptor dimerization (36). Although direct evidence that would support this suggestion is not yet available and our present conclusion is based on inference, it seems to be the only logical and plausible explanation of the experimental data. Transphosphorylation of cytokine receptor-associated kinases is likely a very rapid phenomenon. It should be noted that although initial publications suggested that maximal phosphorylation of JAK2 associated to PRL or GH receptors occurs within 5 min (34), other publications show maximal JAK2 phosphorylation occurring already after 0.5–1 min (37–39). Maximal phosphorylation of JAK2 in MA-10 Leyding cells is also achieved within 0.5 min. Furthermore we have reported that maximal rbPRLR phosphorylation in stably transfected CHO cells occurred already after 0.5 min (40), and another group recently reported that maximal phosphorylation of Stat5A and Stat5B, which occurs downstream from JAK2 activation, was detected after 1 min of exposure (41). Documenting this phenomenon at shorter periods is technically difficult. In conclusion it seems that the classical pharmacological theory that the biological activity is directly related to receptor occupancy does not apply to cytokine receptors in which short term homo- or heterodimerization is sufficient to initiate the transduction of the biological signal. Our present hypothesis explains also the paradoxical agonistic activity of hPRL (G129R), which contrary to expectation exhibited no antagonistic activity in the Nb2 lymphoma cells (42). It also suggests an explanation why the hGH (G120R), which is a site 2 antagonist toward somatogenic receptors, was found to be a potent agonist in Nb2 cells cultured in the absence of zinc ions (43).

4 S. Merchav, personal communication.
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