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THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 275, No. 9, Issue of March 3, pp. 6295–6301, 2000

Printed in U.S.A.

Functional Heterodimerization of Prolactin and Growth Hormone Receptors by Ovine Placental Lactogen*

(Received for publication, October 21, 1999, and in revised form, December 8, 1999)

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Although homo- or heterodimerization are common mechanisms for activation of cytokine receptors, cross-talk between two distinct receptors in this superfamily has been never shown. Here we show a physiologically relevant example indicating that such an interaction does occurs, thus raising the hypothesis that heterodimerization between distinct cytokine receptors may be a novel mechanism contributing to the diversity of cytokine signaling. These findings were documented using both surface plasmon resonance and gel filtration experiments and show that ovine placental lactogen (PL) heterodimerizes the extracellular domains (ECDs) of ruminant growth hormone receptor (GHR) and prolactin receptor (PRLR). We also show that PL or PL analogues that exhibit little or no activity in cells transfected with PRLRs and no activity in cells transfected with ovine GHRs exhibit largely enhanced activity in cells cotransfected with both PRLRs and GHRs. Furthermore, chimeric receptors consisting of cytosolic and transmembrane part of ovine GHR or ovine PRLR and ECDs of human granulocyte-macrophage colony-stimulating factor receptor (GM-CSFR) α or β were constructed. Upon transfection into Chinese hamster ovary cells along with reporter luciferase gene and stimulation by GM-CSF, a significant increase in luciferase activity occurred when GM-CSFR-α-PRLR and GM-CSFR-β-GHR or GM-CSFR-α-GHR and GM-CSRR-β-PRLR were cotransfected. In conclusion, we show that ovine PL is capable of functional heterodimerization of GHR and PRLR and that when their cytosolic parts, coupled to the ECD of GM-CSF receptors, are heterodimerized by GM-CSF, they are capable of transducing biological signal.

Placentas of primates, rodents, and ruminants secrete one or more polypeptide hormones referred to as placental lactogen (PLs)[1] or chorionic somatotropic hormones. They are 22–23-kDa proteins, some of them glycosylated and yielding higher molecular weights, structurally related to pituitary hormones such as growth hormone (GH) and prolactin (PRL) (1, 2). Ovine (o) PL has been purified and characterized by several groups (3–7). It is a nonglycosylated single-chain, 23-kDa protein of 198 amino acids containing 3 S–S bonds. The predicted cDNA sequence reveals 67% identity with bovine PL (bPL), 49% identity with oPRL and 31% identity with mouse PL, whereas the identity with human (h) PL and with either ovine or human GH is lower (25–28%) (8). Recombinant oPL (8, 9) and bPL (10) and recently also caprine PL (11) have been prepared and the recombinant proteins can now be produced in amounts that enable *in vitro* and *in vivo* studies.

One unique property of ruminant PLs, which was observed early on, is their ability to bind to both PRL and GH receptors (1, 2, 12). Comparative binding studies of oPL and oGH to fetal liver microsomes along with the demonstration of oGHR mRNA in fetal liver prompted several research groups to suggest that oGH and oPL bind to identical or at least related, proteins (13–15). Using a similar approach, we previously studied the biological activity of the three ruminant PLs in several *in vitro* bioassays, in which the signal was transduced through heterologous (mouse, rabbit, and human) GHRs (9, 11, 15–19). In all cases, the activity of bPL, oPL, or caprine PL was equal or similar to that of oGH or bGH and in the case of hGH receptors, also to hGH. These experiments were paralleled by protein-interaction studies that showed that oPL and bPL, similar to hGH, are capable of forming 1:2 complex with human and rabbit GHR-ECDs (9, 16).

The mechanism of oPL (and other ruminant PLs) action in homologous systems is, however, less clear. It has been suggested that PLs act as a unique fetal GH, based on findings that ovine fetus responds to ovine or human PL. This response includes stimulation of glycogen synthesis, amino acid transport, cellular proliferation, and insulin-like growth factor-I synthesis. These biological effects in the fetus are only slightly, if at all affected by ovine or human GHs or oPRL, suggesting that oPL may have specific effects (for review see Ref. 2). The way in which oPL signal is initiated remains, however, unknown. It has been suggested that the physiological effects of native oPL in the fetus are mediated through binding to specific PLRs receptors that have low affinities for oGH (20). The K_d for oPL was 0.5 nM, whereas the respective K_d values for oGH and oPRL were approximately 50- and 500-fold higher. However, despite many efforts, these unique receptors have been neither cloned nor identified. The previously reported, partially purified unique oPL receptor (21) turned out to be an artifact.[2]

* This work was supported by USA-Israel Binational Science Foundation Grant 9500327 and USA-Israel Binational Agricultural and Development Fund Grant US-2643-95. 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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[1] The abbreviations used: PL, placental lactogen; PRL, prolactin; PRLR, prolactin receptor; GH, growth hormone; GHR, growth hormone receptor; ECD, extracellular domain; GM-CSF, granulocyte and macrophage colony-stimulating factor; α-PRLR, hGM-CSFR-α-l-oPRLR-pECE; β-PRLR, hGM-CSFR-β-l-oPRLR-pECE; α-GHR, hGM-CSFR-α-oGHR-pECE; β-GHR, hGM-CSFR-β-oGHR-pECE; SPR, surface plasmon resonance; RU, resonance unit; h, human; b, bovine; o, ovine; r, rat; CHO, Chinese hamster ovary; PAGE, polyacrylamide gel electrophoresis.

[2] A. Gertler and M. Freemark, unpublished data.

Experiments executed in homologous mammary gland explants or acini cultures documented that oPL mimics the action of oPRL (9). Recently, we tested the possibility that ruminant PLs transduce their activity through homologous GHRs as well, by comparing their activity in 293-HEK cells transiently transfected with homologous and heterologous GHRs. All three ruminant PLs acted as agonists in several heterologous bioassays (in cells with human or rodent GHRs), whereas in homologous bioassays, in cells transfected with oGHRs, they were not active and even antagonized oGH activity (22). Despite this difference, oGH and PLs bound with similar affinity to the oGHR extracellular domain (ECD), indicating that the binding occurs through site 1 of the hormone. Gel filtration of oPL·oGHR-ECD complex showed a 1:1 stoichiometry, as shown previously for the interaction of bPL and bGHR-ECD (23). Therefore, we proposed that the difference between heterologous and homologous systems originates from the fact that in the latter, ruminant PLs antagonize the activity of oGH because they do not homodimerize oGHRs, whereas in the former they do and thus act as agonists. In view of these findings, we speculated that ruminant PLs may initiate their signaling in four possible ways: (*a*) transducing the signal through homologous PRLRs; (*b*) heterodimerizing homologous GHR through site 1 and PRLR through site 2; (*c*) activating a unique as yet unidentified PLR; and (*d*) activating an as yet unknown variant of GHR, mutated in its ECD such that dimerization of GHR is allowed (22). The present work clearly documents the feasibility of the second possibility.

EXPERIMENTAL PROCEDURES

Materials—Recombinant oPL, oPL T185F, oPL G130R, bPL, bPL G133R, bPL K73F, and nonglycosylated recombinant oGHR-ECD and bPRLR-ECDs were prepared as described previously (9, 10, 15, 17–19, 22, 24). Preparation of oPL K71E and bPL A26W will be described elsewhere. Plasmids encoding full size oGHR and oPRLR in pcDNA3 expression vectors (Invitrogene Co., Leek, The Netherlands), were constructed as described previously (22).[2] Bovine PRLR cDNA in pcDNA1 expression vector (25) was kindly provided by Dr. L. Schuler (University of Wisconsin, Madison, WI). Molecular-weight markers for SDS-PAGE, Dulbecco's modified Eagle's medium and Dulbecco's modified Eagle's medium-Ham's F12 medium, bovine serum albumin (RIA grade) were obtained from Sigma. SDS-PAGE reagents were purchased from Bio-Rad. Fetal calf serum was purchased from Labotal Co. (Jerusalem, Israel), LipofectAMINE was from (Life Technologies, Inc.). A Superdex^{TM}75 HR 10/30 column and SPR reagents including CM5 sensor chips, Hepes-buffered saline, *N*-hydroxysuccinimide, *N*-ethyl-*N*′-(3-diethylaminopropyl)carbodiimide, 2(2-pyridinyldithio)ethanamine hydrochloride, and ethanolamine hydrochloride were obtained from Amersham Pharmacia Biotech.

Determination of Complex Formation—High pressure liquid chromatography gel filtration chromatography on a Superdex^{TM}75 HR 10/30 column was performed with 200-μl aliquots of complexes between the soluble recombinant oGHR-ECD, bPRLR-ECD, and oPL using previously described methods (9, 26). For of preparative chromatography, 600-μl aliquots of preformed complex (the concentration of each component was roughly 27-μM) were injected, and 640-μl samples were collected for electrophoretic analysis. The molecular mass of the complexes was estimated by using several marker proteins with known molecular mass and semi-logarithmic plotting of the molecular mass as a function of retention time. Several consecutive injections of the same markers indicated that the variability of the retention times is not larger than 2–3%. SDS-PAGE was carried out according to Laemmli (27) in a 15% gel. Gels were stained with Coomassie Brilliant Blue R.

Coupling of oPL to a CM Dextran Matrix via Amino Groups—The hormone was covalently linked according to Johnsson *et al.* (28) with few modifications (29). Briefly, after activation with 0.05 M *N*-ethyl-*N*′-(3-diethylaminopropyl)carbodiimide/*N*-hydroxysuccinimide in Hepes-buffered saline (pH 7.4) for 7–8 min, oPL was injected at a concentration of 50 μg/ml in 10 mM sodium acetate buffer (pH 5.4), yielding 2,750 RU of immobilized oPL. Nonreacted sites were blocked with an 8-min injection of 1 M ethanolamine hydrochloride at pH 8.5, and binding capacity was checked by repeated injections of 5 M rabbit PRLR-ECD (26) in Hepes-buffered saline. Regeneration was performed by a 1-min injection of 5 M guanidine-HCl.

Kinetics Measurements of R-ECD-Hormone Interactions—All experiments were performed at a flow rate of 5 μl/min in Hepes-buffered saline at 25 °C. Once the oPL was covalently immobilized through amino-group coupling, each R-ECD was injected for 6 min and then washed out for 10 min prior to regeneration.

Data Analysis and Calculation of Kinetics Constants—BIAcore incorporated software (BIA Evaluation and BIA Simulation) allowed us to: (*a*) fit experimental curves to 1:1 or 1:2 dissociation models and calculate the probabilities of each being the most accurate representation of reality and (*b*) calculate k_{off} constants with standard deviations.

In Vitro Bioassays in Transiently Transfected 293-HEK Cells—The bioassays were carried out in 293 cells as described previously (30). Briefly, 293-HEK cells were seeded in six-well plates in rich medium. After 4–8 h, the cells were transfected with oPRLR-pcDNA3, alone or in combination with oGHR-pcDNA3. The total amount of DNA was equalized using pcDNA3 plasmid without insert. In parallel, cells were transfected with LHRE-TK-luc, a plasmid bearing six repeats of the rat β-casein STAT5-responsive sequence upstream of a thymidine kinase minimal promoter linked to a luciferase reporter gene (31), and pCH110, a plasmid encoding β-galactosidase activity (Amersham Pharmacia Biotech). Transfection was carried out using the calcium-phosphate procedure described elsewhere (32, 33), with minor modifications. After 24 h, the cells were transferred to serum-free medium, hormonal treatment was added, and cells were incubated for an additional 18 h. Enzymatic activity was measured as described elsewhere (31, 34). The results were expressed as fold induction, and the ratio of stimulated to nonstimulated cells after luciferase activity was normalized by correcting for β-galactosidase activity, to take into account the transfection efficiency.

Preparation of Chimeric Receptors Consisting of the ECD of Human Granulocyte and Macrophage Colony-stimulating Factor Receptor (hGM-CSFR) and of Transmembrane and Cytosolic Domains of oGHR or oPRLR—Four chimeric constructs were prepared: the ECD of the α or β subunit of the hGM-CSF receptor (hGM-CSFR-α ECD, hGM-CSFR-β ECD) was fused to the transmembrane and cytosolic domains of the long form oPRL (oPRLR) or growth hormone (oGHR) receptor. The four DNA fragments were obtained by polymerase chain reaction with the following primers and templates: GGG CCC CTG CAG ATG CTT CTC CTG GTA ACA AGC (5′ primer), GAA TTC AAG CTT CTC GAG CCC GTC GTC AGA ACC AAA TTC (3′ primer), and the hGM-CSFR-α cDNA (35); GGG CCC CTG CAG ATG GTG CTG GCC CAG GGG CTG (5′ primer), GAA TTC AAG CTT CTC GAG CGA CTC GGT GTC CCA GGA GCG (3′ primer), and the hGM-CSFR-β cDNA (36); GGG CCC CTG CAG CTC GAG TTT CCA TGG TTC TTA ATT ATT (5′ primer), GAA TTC AAG CTT TCT AGA CTA CGG CAT GAT TTT GTT CAG (3′ primer), and the oGHR cDNA (37); and GGG CCC CTG CAG CTC GAG ACA AGC ATG TGG ATC TTT GTG (5′ primer), GAA TTC AAG CTT TCT AGA CTA AGG CAG GGC TGG CGG (3′ primer), and the long oPRLR cDNA (38). Sequencing confirmed the absence of misincorporation by *Taq* polymerase. After digestion by *Hin*dIII and *Xho*I (ECD) or *Xho*I and *Xba*I (TMI), each ECD was ligated to each TMI in the presence of *Hin*dIII/*Xba*I-digested eucaryotic expression vector pECE (39). This resulted in four chimeric constructs: hGM-CSFR-α-l-oPRLR-pECE (α-PRLR), hGM-CSFR-β-l-oPRLR-pECE (β-PRLR), hGM-CSFR-α-oGHR-pECE (α-GHR), and hGM-CSFR-β-oGHR-pECE (β-GHR).

Determination of Biological Activity Induced by GM-CSF through Chimeric Receptors—The β-galactosidase and luciferase assays have been described previously (Refs. 34 and 31, respectively). Briefly, CHO-K1 cells were seeded in four 60-mm dishes in rich medium. The next day, the cells were starved for 16 h by incubation in GC3 medium. On the third day, the cells were transfected using LipofectAMINE with pCH110 along with LHRE-TK-luc, and with one or two of the four afore described chimera constructs. Transfected cells were subsequently incubated for 24 h in the presence (two plates) or absence (two plates) of 100 ng/ml hGM-CSF in GC3 medium. The plates were washed with phosphate-buffered saline, and the enzymatic activity was determined as described previously (31, 34). The results were expressed as fold induction, after the luciferase activity was normalized by correcting for β-galactosidase activity, as explained earlier.

RESULTS

Interaction of oPL with oGHR-ECD and bPRLR-ECD—Recombinant bPRLR-ECD was chosen because this protein is almost identical (94.5% identity and 96% similarity) to oPRLR-ECD and both ovine and bovine full-size PRLRs gave identical

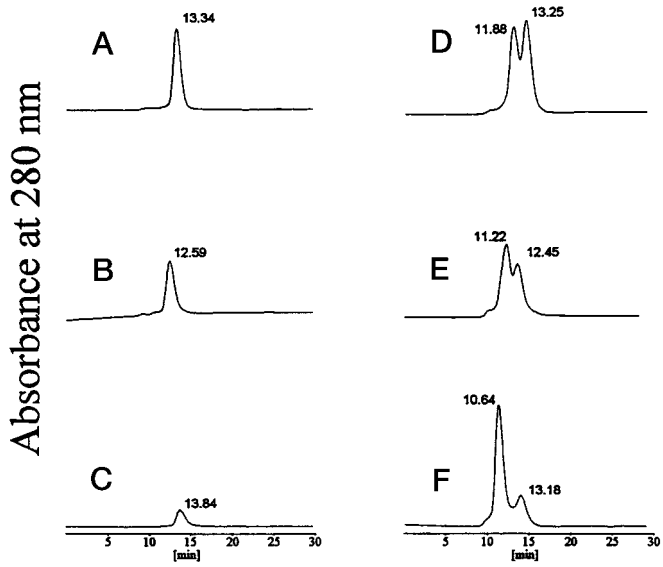

FIG. 1. **Gel filtration of oGHR-ECD (*A*), bPRLR-ECD (*B*), oPL (*C*), a complex of oPL preformed with a 2-fold molar excess of bPRLR-ECD (*D*), a complex of oPL preformed with twofold molar excess of oGHR-ECD (*E*), or a complex of oPL preformed with approximately equal molar quantities of both oGHR-ECD and bPRLR-ECD (*F*).** Complex formation was carried out during a 20–30-min incubation at room temperature in TN buffer, and then aliquots (200 μl) of the incubation mixture were applied to a Superdex^{TM}75 HR 10/30 column, pre-equilibrated with the same buffer. The initial hormone concentration (2 μM) was constant in all cases. The column was developed at room temperature at 0.8 ml/min, and protein concentration was monitored by absorbance at 280 nm. Each experiment was conducted at least three times.

biological response to both bPL and oPL.[3] The interaction of oPL with bPRLR-ECD and oGHR-ECD was studied by two independent methods, namely, gel filtration and SPR in a Biacore apparatus. Gel filtration revealed that oPL forms only 1:1 complex with each ECD even at 2:1 (Fig. 1, *D* and *E*) excesses of the respective R-ECDs. However, when oPL, oGHR-ECD, and bPRLR-ECD were mixed in almost equal molar ratios, a complex with a higher molecular mass, corresponding to a heterodimeric complex along with a small excess of oPRLR-ECD, was observed (Fig. 1*F*). This complex was quite stable at μM concentrations (Fig. 2, *A* and *B*) but upon progressive dilution to nM concentrations underwent partial (Fig. 2*C*) or full (Fig. 2*D*) dissociation. The order of addition, or preincubation of oPL with one of the ECDs prior to addition of the other, had no effect on the gel filtration profile (not shown). The protein peak corresponding to this complex was isolated (Fig. 3, *bars 6–8*) and analyzed by SDS-PAGE. As judged by the intensity of the bands stained with Coomassie Blue (Fig. 3, *inset*), it consists of three components: oPL, oGHR-ECD, and bPRLR-ECD, in almost equal quantities. As shown by SDS-PAGE, the small peak with the lower molecular mass (Fig. 3, *bar 10*) was indeed bPRLR-ECD, as predicted. No complex was formed by incubation of oGHR-ECD and PRLR-ECD in the absence of oPL (not shown).

The results obtained from the gel filtration experiments were further validated by SPR analysis. First, binding capacity was checked with rabbit PRLR-ECD revealing the immobilization of active 2,750 RU of oPL. Then 1 μM solutions of either oGHR-ECD or bPRLR-ECD were injected for 20 min, followed by flushing with buffer for another 20 min (Fig. 4*A*). The dissociation of each R-ECD was analyzed with Bioevaluation (BIA) Software. In the case of oGHR-ECD, which at that concentration failed to bind over 2,700 RU (stoichiometry, 1:1), the dissociation kinetics clearly showed a good fit to a one-site interaction model, and the calculated k_{off} value (mean ± S.D., $n = 3$) was $3.4 \pm 0.30 \times 10^{-4}$ min^{-1}. In contrast, although bPRLR reached a maximum of about 2,200 RU, the dissociation kinetics was indicative of a two-site interaction model with loose binding at each site, which, as shown by us previously is characteristic of homologous interaction with PRLRs (18, 19, 29). The two dissociation constants (mean ± S.D., $n = 3$ or 4) were, respectively, $k1_{off} = 3.08 \pm 0.33 \times 10^{-3}$ min^{-1} and $k2_{off} = 4.9 \pm 0.15 \times 10^{-2}$ min^{-1}. We concluded therefore that in the first case (oGHR-ECD), the homodimer is not conspicuous in the gel filtration profile (Fig. 1*D*) because it is not assembled, whereas the bPRLR-ECD homodimer is too unstable to be observed (Fig. 1*E*).

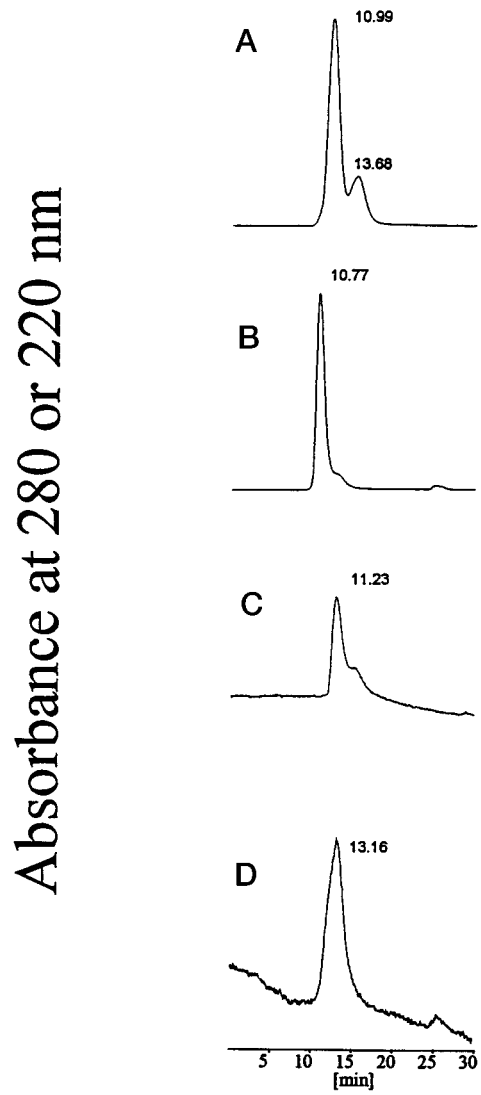

FIG. 2. *A*, gel filtration of 1:1:1 complex of oPL·oGHR-ECD·bPRLR-ECD preformed by incubation of all three components at concentrations of 27 μM. *B*, gel filtration of the complex-containing peak (6.3 μM) obtained in *A*. *C*, as in *B* but after 10-fold dilution with TN buffer. *D*, as in *B* but after 100-fold dilution with TN buffer. The protein concentration in the eluate was monitored by absorbance at 280 nm (*A–C*) or 220 nm (*D*). For other details see the legend to Fig. 1.

The next experiment was aimed at showing the occurrence of oPL-induced heterodimerization (Fig. 4*B*). After the first 20-min injection of 1 μM oGHR-ECD, which gave results identical to those shown in Fig. 4*A*, the chip was flushed for 30 s with buffer, followed by a second injection using oGHR-ECD (*trian-*

[3] D. Helman, A. Herman, and A. Gertler, unpublished data.

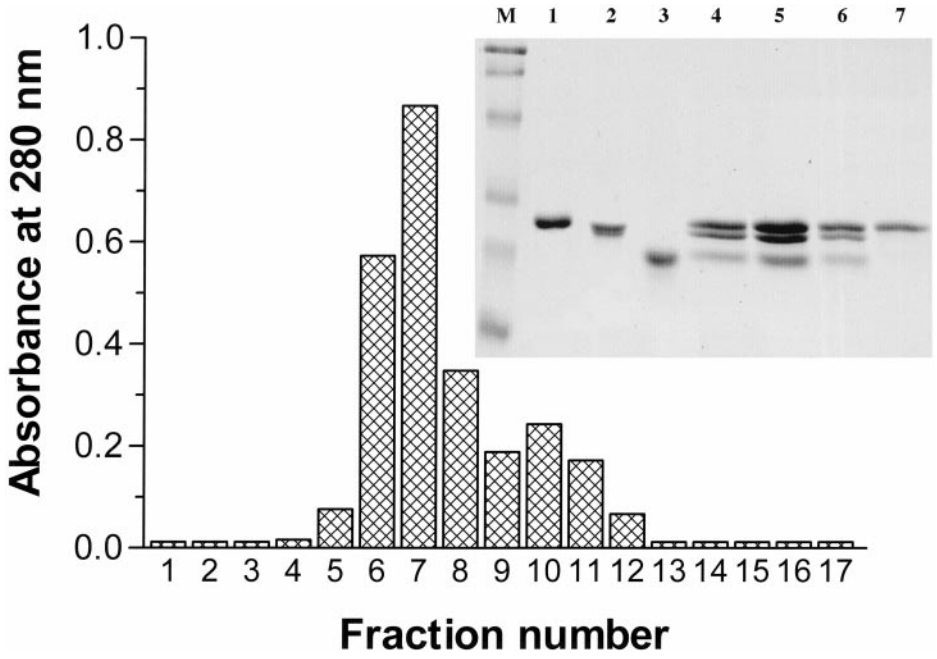

FIG. 3. **Gel filtration of 1:1:1 complex of oPL·oGHR-ECD·bPRLR-ECD (total 600 μl) preformed by incubation of all three components at 27 μM concentrations.** 6 min after injection, 640-μl samples were collected for electrophoretic analysis. The protein concentration was determined manually by reading at 280 nm. For calculation of the molecular mass the column was calibrated with bovine serum albumin (66 kDa), egg albumin (45 kDa), extracellular domain of hGH receptor (28 kDa), and ovine placental lactogen (23 kDa). For other details, see legend to Fig. 1. *Inset*, SDS-PAGE (15% gel), molecular mass markers from the *top* to the *bottom*: 97, 66, 45, 31, 21.5, and 14.5 kDa (*lane M*), bPRLR-ECD (*lane 1*), oGHR-ECD (*lane 2*), oPL (*lane 3*), and of 25-μl aliquots obtained from fractions 6–8 (*lanes 4–6*) and 10 (*lane 7*). Gels were stained with Coomassie Brilliant Blue R.

gles), bPRLR-ECD (*circles*), or buffer (*squares*). Each experiment was performed three or four times. In the first case (oGHR-ECD → oGHR-ECD), the second injection just compensated for the dissociation, but the second site remained unoccupied. This was in agreement with the results shown in Fig. 4*A* and in both experiments, the level of 2,700 RU was not exceeded. In the second case (oGHR-ECD → bPRLR-ECD), the PRLR-ECD docked into the second site which was unoccupied by oGHR and reached a level of 4,100 RU. Following flushing with buffer it rapidly dissociated, and the dissociation kinetics were indicative of a two-site interaction model. Calculation of the respective k_{off} values showed the same dissociation rate (mean ± S.D., $n = 3$ or 4) for oGHR as in homogeneous docking ($k1_{off} = 3.30 \pm 0.35 \times 10^{-4}$ min^{-1}). The binding of bPRLR-ECD was slightly more stable ($k2_{off} = 1.26 \pm 0.27 \times 10^{-2}$ min^{-1}), as compared with the binding of bPRLR-ECD to the second site when the first site was also occupied by bPRLR-ECD (Fig. 4*A*). In the third case (oGHR-ECD → buffer), the kinetics of dissociation was identical to that shown in Fig. 4*A*. It can thus be concluded that the heterodimer is more stable than the bPRLR-ECD homodimer, in agreement with the aforementioned gel filtration experiments.

The Biological Response to oPL, or oPL and bPL Analogues, in 293-HEK Cells Cotransfected with oPRLR and oGHR—We previously showed that in 293 cells transiently transfected with full-size oGHR and a reporter luciferase gene (with STAT5-responsive LHRE), oPL is not active and acts as an oGH antagonist by blocking the site 1 of oGHR (22). In contrast, in 293-HEK cells transiently transfected with either oPRL or bPRL full-size receptors, oPL mimicked oPRL and induced luciferase expression (22).[4] The aim of the present experiment was to test whether cotransfection of both oGHR and oPRLR augments the activity observed in cells transfected with oPRLR only. For this purpose, the cells were transfected with expression vector encoding the full-size oPRLR (0.1 μg/well) alone or with the same amount of expression vector encoding the full-size oGHR. The cells were then stimulated with oPL, or several oPL or bPL analogues, at several concentrations. Cotransfection clearly increased the hormone-induced

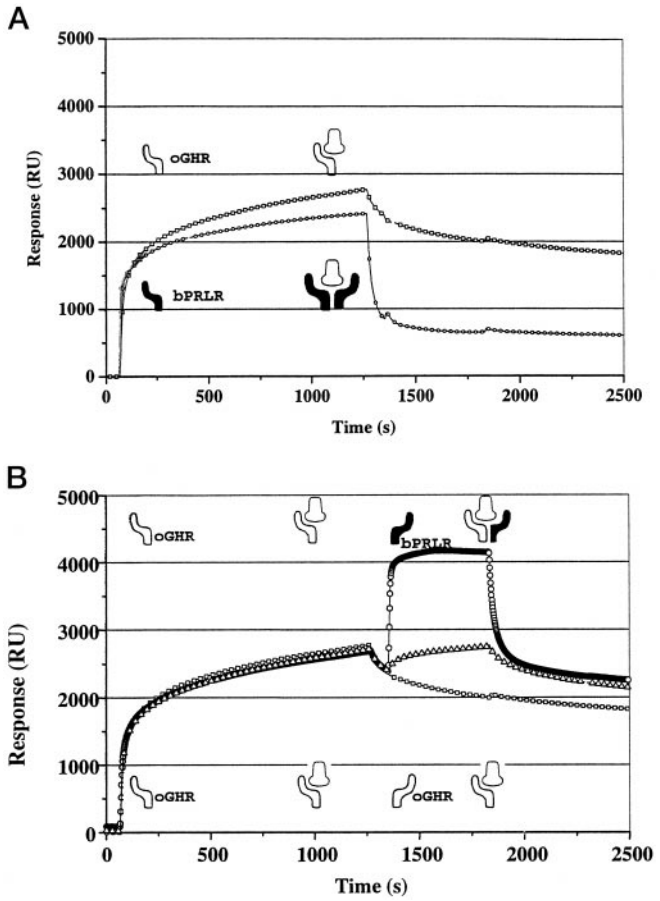

FIG. 4. **SPR analysis of complexes of oPL·oGHR-ECD and oPL·bPRLR-ECD (*A*) and of heterodimeric (1:1:1) complex composed of oPL·oGHR-ECD·bPRLR-ECD (*B*).** In *A*, after the first 20-min injection of 1 μM oGHR-ECD (*squares*) or of bPRLR-ECD (*circles*), the chip was flushed for another 20 min with buffer. In *B*, after the first 20-min injection of 1 μM oGHR-ECD the chip was flushed for 30 s with buffer followed by a second injection (lasting 9.5 min) using oGHR-ECD (*triangles*), bPRLR-ECD (*circles*), or buffer (*squares*). Subsequently, in all three experiments, the chip was flushed with buffer for another 10 min. Symbols represent the molecular interactions occurring during the course of the experiment.

[4] D. Helman, A. Herman, J. Paly, O. Livnah, P. A. Elkins, A. M. Devos, J. Djiane, and A. Gertler, submitted for publication.

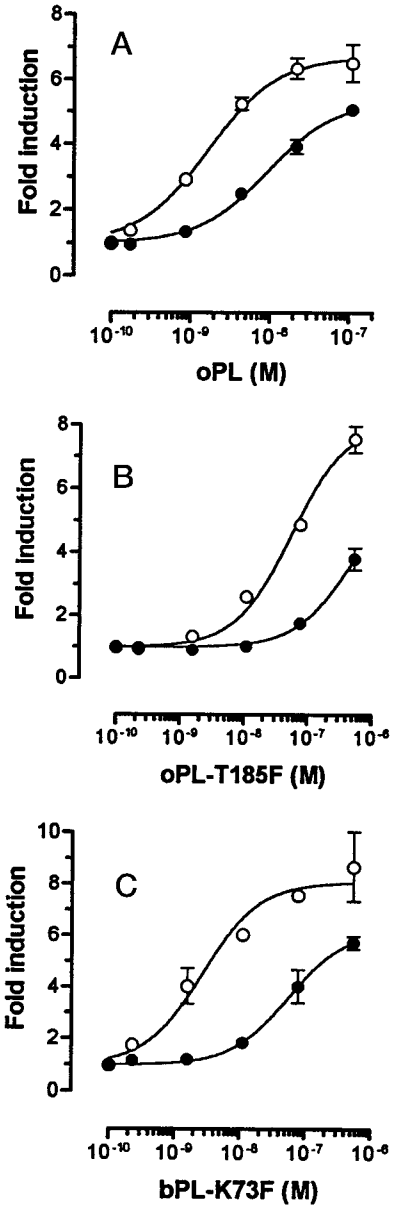

FIG. 5. **Ability of oPL (*A*), oPL T185F (*B*), and bPL K73F (*C*) to promote LHRE-tk-luc transcription in 293 cells transiently transfected with ovine PRLRs (*filled circles*) or ovine PRLRs and GHRs (*open circles*).** For other details, see text.

activity as compared with cells transfected with PRLR only (Fig. 5). In the case of oPL (Fig. 5*A*), the increase resulted in an over 5-fold decrease in the EC_{50} value, from 9.5×10^{-9} to 1.7×10^{-9} M. Even more pronounced augmentation was observed with oPL T185F and bPL K73F analogues (Fig. 5, *B* and *C*), which were much weaker agonists than oPL or bPL[4] in 293 cells expressing oPRLR. Following cotransfection with both oPRLR and oGHR, the respective EC_{50} values (calculated by extrapolation) decreased from 4.7×10^{-7} to 0.55×10^{-7} for oPL T185F and from 5.5×10^{-8} M to 0.27×10^{-8} for bPL K73F. In contrast, other analogues, such as oPL G130R, oPL K71E, bPL G133R, and bPL A26W, which have no activity in 293 cells transfected with oPRLR, were also inactive in cells transfected with both oPRLR and oGHR (not shown). No such augmentation was observed when the cotransfected cells were stimulated with either oGH or oPRL (not shown). Furthermore, the augmentation observed in cells cotransfected with both oPRLR and oGHR could be abolished in a dose-dependent manner by adding oPL G130R (not shown), a nonactive analogue, which, as documented previously, competes with oPL for binding to oGHR but not to bPRLRs (22).[4] In cells stimulated with 4.3×10^{-9} M oPL, 8- and 20-fold excess of oPL G130R, abolished 50 and 95%, respectively, of the augmentation.

Transduction of the hGM-CSF Signal in Chimeric Receptors by Controlled Dimerization of Transmembrane and Cytoplasmic Domains of the oGH and oPRL Receptors—To evaluate the ability of the cytoplasmic domains of oGHR and of the long form of oPRLR to transmit the signal to a target gene when they are associated in a heterodimeric complex, their coding sequences were fused inframe, downstream of the coding sequence of the ECD of the α or β subunits of hGM-CSF receptor, and the respective chimeric constructs were prepared. Because only α- and β-subunit associations create a high affinity receptor for hGM-CSF (35), activation of a target gene in cells transfected with two receptor constructs, one bearing the α-ECD and the other one the β-ECD can only be attributed to ligand-induced α/β association. One or all combinations of two of the four chimeras were tested in the functional assay described under "Experimental Procedures" for their ability to promote transcription of a target gene. Results of these experiments are summarized in Fig. 6. As shown, when chimeric constructs α-PRLR and β-PRLR were cotransfected, addition of hGM-CSF resulted in a more than 3-fold increase in target promoter activity (*lane 1*). In contrast, no induction was detected in CHO cells transfected with only one of the two constructs (*lanes 5* and *6*), hence indicating that the transcriptional activity stimulated by the dimerized oPRLR cytoplasmic domains resulted from the α/β association and not from the α/α or β/β associations. Similar results were obtained when chimeric constructs α-GHR and β-GHR were cotransfected with an even higher (more than 5-fold) level of transcription stimulation (*lane 2*). Induction was not detected in CHO cells transfected with only one construct (*lanes 7* and *8*). Similar induction was also observed when heterodimers were allowed to form by transfecting CHO cells with either α-PRLR and β-GHR (*lane 3*) or with β-PRLR and α-GHR (*lane 4*) and stimulating with hGM-CSF. This clearly demonstrated that hormone-induced association of the cytoplasmic domain of PRLR with the cytoplasmic domain of GHR in the same species results in a molecular heterodimer with the capacity to transmit the hormonal signal to a target gene comparable to the two corresponding homodimers. Identical results were obtained when plasmids bearing natural promoters, such as rat β-casein, rabbit β-lactoglobulin, or Spi, upstream of a reporter gene, were used (not shown).

DISCUSSION

The availability of pure recombinant oPL, bPRLR-ECD, and oGHR-ECD enabled a direct study of their interaction by two independent methods. Gel filtration experiments clearly indicated that a complex, consisting of three components, was formed. This is evidenced by the fact that when oPL was incubated with excess of either oGHR or oPRLR, only 1:1 complex was formed and excesses of the respective ECDs could be seen (Fig. 1). Moreover, when a triple complex with a higher molecular mass was formed and isolated, its three components could be resolved by SDS-PAGE (Fig. 3). Calculation of the molecular mass of the heterodimeric complex yielded a value of 62 kDa, *versus* the expected value of 73 kDa predicted from the 1:1:1 stoichiometry. This discrepancy could result from formation of a more compact structure as suggested by Fritz *et al.* (40), who studied trypsin-trypsin inhibitor interactions, and as also observed in our previous studies of interactions between rabbit or rat PRLR-ECDs and bPL (26, 41). Semi-quantitative estimation indicated that the heterodimeric complex is stable at micromolar concentrations and dissociates upon dilution.

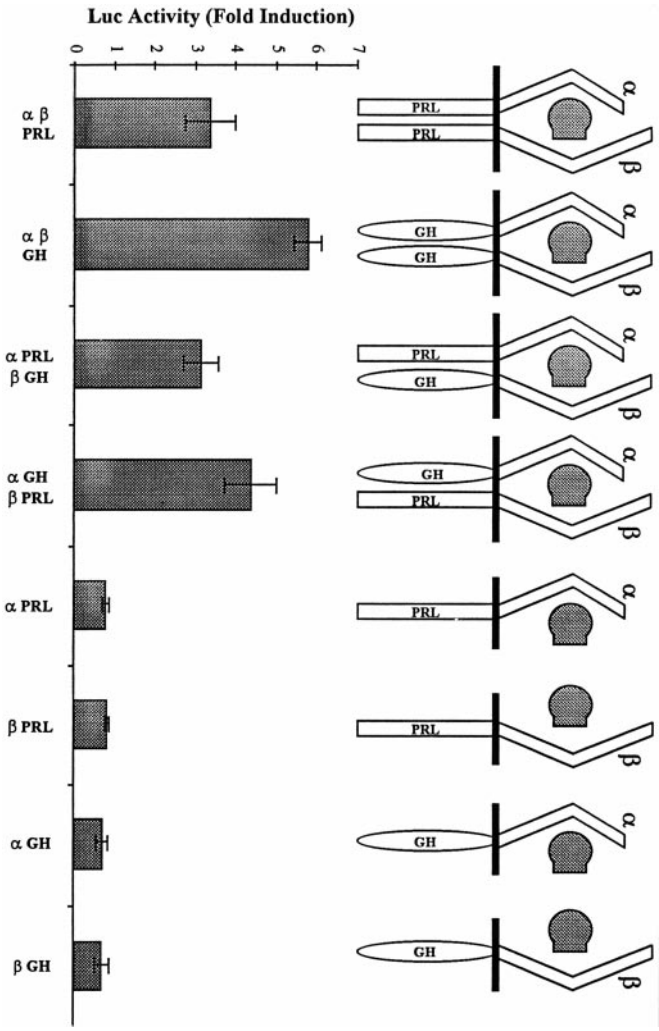

FIG. 6. **Evaluation of the capacity of cytoplasmic domains of the long form of oPRLR or GHR to activate transcription of a target promoter.** The *upper part* of the figure shows different combinations of the four chimeric proteins made up of the ECD of the α or β subunits of hGM-CSF receptor fused to the transmembrane and cytoplasmic domains of PRLR or GHR, expressed at the surface of transfected CHO cells, and bound to one molecule of hGM-CSF. The *horizontal bar* is the cell membrane, α and β refer, respectively, to the ECDs of α and β subunit of the hGM-CSF receptor, and *PRL* and *GH* refer to the transmembrane and cytoplasmic domains of the PRLR and GHR. The ability of each combination to promote LHRE-tk-luc transcription in CHO cells (mean ± S.E.), following the addition of hGM-CSF, is given in the *lower portion* of the figure. From *left* to *right*, *bar 1,* PRLR homodimer; *bar 2*, GHR homodimer; *bars 3* and *4*, PRLR/GHR heterodimers; *bars 5* and *6*, PRLR monomers; *bars 7* and *8*, GHR monomers.

Because during the course of gel filtration the injected material undergoes 5–10-fold dilution, the actual concentration of the complex could not be accurately determined. However as the interaction with bPRLR-ECD is obviously weaker than that with oGHR-ECD (see below), the lower-than-expected molecular mass could also result from a partial and gradual dissociation occurring during the course of the chromatography, as also indicated by the right-skewed peak of the complex observed in dilution experiments (Fig. 2). It should be noted that the observed molecular mass (50.5 kDa) of the 1:1 oPL·oGHR-ECD complex was very close to the predicted value (51.1 kDa), unlike the 1:1 complex of oPL·bPRLR-ECD in which the respective values were 39.9 and 44.5.

SPR studies also clearly indicated the formation of a heterodimeric complex formed by consecutive binding of oGHR-ECD and bPRLR-ECD to immobilized oPL (Fig. 4). In view of the higher affinity for oGHR-ECD, its displacement by excess of bPRLR-ECD is extremely unlikely, although a study with the latter indicated that formation of transient 2:1 oPRLR-ECD·oPL does occur. As mentioned earlier, this complex could not be detected by gel filtration because of its rapid dissociation to the 1:1 form. This result further emphasizes the limitations of gel filtration or classical binding experiments in predicting biological activity in the cases of transient complexes. The SPR results support the suggestion of Wells *et al.* (42) that formation and dissociation of a 2:1 receptor-hormone complex are carried out sequentially. Our results suggest, therefore, that site 1 of the oPL is occupied by oGHR-ECD and site 2 by bPRLR-ECD and not *vice versa.* Calculations of the respective dissociation constants fully support this hypothesis. Similar results using SPR methodology have also been obtained by using bPL, bGHR-ECD, and bPRLR-ECD.[5]

To test whether heterodimerization of oGHR and oPRLR leads to the initiation of biological signal, two experiments were performed. In 293 cells in which oPL and some oPL analogues can activate oPRLRs,[3] cotransfection of both receptors clearly augmented the response as compared with transfection with oPRLR only (Fig. 5). Those experiments were performed with oPRLR-cDNA as the limiting factor (data not shown). Because oPL is unable to homodimerize oGHRs and evokes no biological response in cells transfected with oGHR (22), the only logical explanation is to attribute the enhancement of the biological signal to heterodimerization of oGH and oPRL receptors. The 5–20-fold decrease in the EC_{50} values (Fig. 5) obtained in cotransfected cells, and the finding that oPL G130R analogue abolished the augmentation is fully compatible with this conclusion.

The finding showing that heterodimerization of oGH/oPRL receptors is productive was also clearly documented by using the chimeric receptor consisting of cytosolic and transmembrane parts of oGHR or oPRLR and ECDs of GM-CSFR-α or β (Fig. 6). We cannot explain, however, why the GHR homodimer stimulated target promoter transcription more efficiently than did the PRLR homodimer. Similarly, the α-GHR/β-PRLR heterodimer promoted higher transcriptional activity than did the α-PRLR/β-GHR heterodimer. Interestingly, these differences were maintained when promoters other than LHRE were used (not shown). The fact that JAK2, the tyrosine kinase responsible for signal transduction downstream of the receptor, is constitutively associated with PRLRs (43), whereas it associates with GHRs only upon hormonal stimulation (44), suggests a direction for future investigations.

The next obvious question is whether the events observed in our protein-protein interaction and *in vitro* studies indeed reflect the physiological situation in which signal transduction occurs as a result of ruminant GHR/PRLR heterodimerization. Numerous studies (45–50) in which the effect of either oPL or bPL was studied in heterologous (rodent and human) systems are irrelevant because in these cells ruminant PLs homodimerize GHRs. Binding studies to GHRs (14, 51, 52) are also not indicative of biological activity, because they are likely to represent binding of oPL through site 1 only (22, 30). A suitable study model would therefore be a cell expressing both ruminant PRLR and GHR, in which unique biological response could be evoked by homologous PL, but not by GH or PRL. A limited number of studies have shown feasibility of this hypothesis. One such study tested the effect of oPL on glycogen metabolism, in a homologous system of cultured ovine fetal hepatocytes. Ovine PL stimulated a dose-dependent increase in [^{14}C]glucose incorporation into glycogen and in total cellular glycogen content, whereas the effects of oGH and

[5] J. C. Byatt, J. J. Shieh, and N. R. Staten, personal communication.

oPRL were only 12 and 4%, respectively (20). In more recent studies Gluckman and co-workers (53) compared the action of oPL and bGH *in vivo*. They demonstrated that oPL has a distinct effect on food intake (53) and on the expression of insulin-like growth factor-I and insulin-like growth factor-binding protein 3 (54). The same group also reported that in well fed postnatal lambs, blood glucose and the insulin/glucose ratio were significantly ($p < 0.05$) elevated in the bGH+oPL group, whereas they were not significantly altered by treatment with either bGH or oPL alone (55). *In vivo* experiments in lactating cows have also indicated that the effect of bPL may be distinct from that of bGH (56). We have recently found that the mammotropic effect of oPL and oGH in pseudopregnant ewes is similar, although only oGH increases expression of insulin-like growth factor-I (57). oGH and oPL also had profound, similar, and statistically significant growth-stimulating effects, enhancing lamb growth by 10–25%, whereas PRL is known to be inactive as a growth stimulant. In contrast, the galactopoietic effect of oGH was considerably stronger than that of oPL, whereas oPRL was inactive.[6] It was also observed that oPL stimulates both uterine milk protein and osteopontin expression in the endometrial glandular epithelium, whereas oGH only stimulates uterine milk protein expression, furthers indicating a unique effect of oPL.[7]

In conclusion, because: (*a*) the existence of unique PL receptor is highly questionable and (*b*) the previously reported, putative, partially purified unique oPLR (21) turned-out to be an artifact,[3] the only feasible explanation for oPL activity that is distinct from that of PRL and GH is heterodimerization of homologous GHR and PRLR. Although the present work deals specifically with GHR and PRLR it may have wider implications. Receptor dimerization is frequently an initial event in cytokine signaling (58–60); however, it is not known whether two distinctly different cytokine receptors can form a functional complex. Here we show a physiologically relevant example, indicating that such an interaction does occur and thus raising a hypothesis that heterodimerization between distinct cytokine or at least between PRL and GH receptors may be a novel mechanism contributing to the diversity of cytokine signaling.

Acknowledgments—We are grateful to Prof. E. B. Leof, from Mayo Clinic (Rochester, MN) for providing us with the vector encoding the GM-CSF receptor and Prof. P. A. Kelly, Dr. V. Goffin, and Dr. A. Tchelet from INSERM U366 (Paris, France) for providing us with the 293 cells and the vectors encoding luciferase and receptors.

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Functional Heterodimerization of Prolactin and Growth Hormone Receptors by Ovine Placental Lactogen

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J. Biol. Chem. 2000, 275:6295-6301.
doi: 10.1074/jbc.275.9.6295
