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Ovine Placental Lactogen-Induced Heterodimerization of Ovine Growth Hormone and Prolactin Receptors in Living Cells Is Demonstrated by Fluorescence Resonance Energy Transfer Microscopy and Leads to Prolonged Phosphorylation of Signal Transducer and Activator of Transcription (STAT)1 and STAT3

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HEK-293T cells transiently transfected with ovine (o) GH receptor (GHR) and prolactin receptor (PRLR) constructs respectively tagged downstream with cyan or yellow fluorescent proteins were used to study ovine placental lactogen (oPL)-stimulated heterodimerization by fluorescence resonance energy transfer (FRET) microscopy. The oPL-stimulated transient heterodimerization of GHR and PRLR had a peak occurring 2.5–3 min after oPL application, whereas oGH or oPRL had no effect at all. The results indicate none or only little dimerization occurring before the hormonal stimulation. The effect of heterodimerization was studied by comparing activation of Janus kinase 2, signal transducer and activator of transcription (STAT)1, STAT3, STAT5, and MAPK in Chinese hamster ovary cells stably transfected with chimeric genes encoding receptors consisting of cytosolic and transmembrane parts of oGHR and oPRLR, extracellular domains of human granulocyte and macrophage colony-stimulating factor (hGM-CSF) receptor α or β, and cells transfected with the two forms (α or β) of PRLR and GHR. Functionality of those proteins was verified by hGM-CSF-induced phosphorylation of both intracellular PRLR and GHR domains and hGM-CSF-induced heterodimerization was documented by chimeric receptor coimmunoprecipitation. Homodimerization or heterodimerization of PRLRs and GHRs had no differential effect on activation of STAT5 and MAPK. However, heterodimerization resulted in a prolonged phosphorylation of STAT1 and in particular STAT3, suggesting that the heterodimerization of α-oGHR and β-oPRLR is able to transduce a signal, which is distinct from that occurring on homodimeric associations. (*Endocrinology* 144: 3532–3540, 2003)

PLACENTAS OF PRIMATES, rodents, and ruminants secrete polypeptide hormones known as placental lactogen (PLs). These 22- to 23-kDa proteins, some of them glycosylated, are structurally related to pituitary hormones such as GH and prolactin (PRL) (1, 2). Ovine (o) PL, a nonglycosylated single-chain 23-kDA protein, has been purified and characterized by several groups (3–7), and recombinant oPL has been prepared by us (8) and others (9). It is well documented that oPL, as well as other ruminant PLs, are capable of binding to both PRL and GH receptors (GHRs) (1, 2, 10). A 1:2 complex between oPL and the extracellular domain (ECD) of the rat PRL receptor (PRLR) has been crystallized and its three-dimensional structure elucidated (11). However, in contrast to heterologous interactions with mouse, rabbit, and human (h) GHRs that lead to homodimerization and subsequent biological activity (8, 12–17), oPL and other ruminant PLs are unable to homodimerize the ruminant GHRs and form only 1:1 complex with the respective GHR-ECDs (18); consequently, they are devoid of somatogenic activity in homologous species.

Despite extensive effort, the existence of a unique PL receptor has never been documented because the previously reported, putative, partially purified oPL receptor (19) turned out to be an artifact (Gertler, A., and M. Freemark, unpublished data). Therefore, we proposed that oPL possesses a unique property, distinct from that of PRL and GH, resulting from heterodimerization of homologous GHR and PRLR. Our hypothesis was documented using both surface-plasmon-resonance and gel-filtration experiments, which showed that, indeed, oPL forms a heterotrimeric complex by binding both GHR through its site 1 and PRLR through its site 2 (20). We also showed that oPL exhibits low activity in cells transfected with PRLRs, no activity in cells transfected

Abbreviations: CFP, Cyan fluorescent protein; CHO, Chinese ovary hamster; ECD, extracellular domain; FRET, fluorescence resonance energy transfer; GHR, GH receptor; GM-CSF, granulocyte and macrophage colony-stimulating factor; HEK, human embryonic kidney; JAK, Janus kinase; LUC, luciferase; PL, placental lactogen; PRL, prolactin; PRLR, PRL receptor; STAT, signal transducer and activator of transcription; TM, transmembrane; YFP, yellow fluorescent protein.

with oGHRs but significantly greater activity in cells cotransfected with both receptors. Furthermore, chimeric receptors consisting of cytosolic and transmembrane (TM) parts of oGHR or oPRLR and ECDs of human granulocyte and macrophage colony-stimulating factor receptors (hGM-CSFRs) α or β have been constructed. Upon transient transfection into Chinese ovary hamster (CHO) cells along with a reporter luciferase (LUC) gene, stimulation by hGM-CSF led to a significant increase in LUC activity in cells cotransfected with hGM-CSF-α-oPRLR and hGM-CSF-β-oGHR or hGM-CSF-α-oGHR and hGM-CSF-β-oPRLR (20). These results indicate that when their cytosolic parts, coupled to the ECD of hGM-CSFRs, are heterodimerized, these chimeric receptors are capable of transducing a biological signal (20).

Although these data suggest that oPL signaling in a homologous system may result from its ability to not only homodimerize PRLRs but also via heterodimerization of GHR and PRLR, to date proof of such heterodimerization occurring in living cells has been indirect. Furthermore, our experiments did not provide any indication of whether the signal induced by heterodimerization leads to any unique effects, different from those caused by homodimerization of GHRs or PRLRs. To answer those queries, two experimental approaches were used in the present work. First, to prove that oPL induces heterodimerization of GHR and PRLR in living cells, we prepared oGHR and oPRLR constructs tagged downstream with cyan (CFP) or yellow (YFP) fluorescent proteins and proved that the tagging does not affect their biological activity (21). Then we used these constructs to study oPL-induced fluorescence resonance energy transfer (FRET) in living cells. Second, we prepared three clones of CHO cells stably transfected with the chimeric receptors. Two homodimeric clones expressed chimeric receptor composed of the cytosolic and TM parts of oGHR and the ECD of hGM-CSFRα or -β, or the cytosolic and TM parts of oPRLR and ECD of hGM-CSFR α or β and one heterodimeric clone expressed a chimeric receptor of the cytosolic and TM parts of oGHR and the ECD of hGM-CSFR-α and cytosolic and TM parts of oPRLR and the ECD of hGM-CSFR-β. These clones were used to identify unique signaling resulting respectively from homodimerization of oGHRs or oPRLRs from heterodimerization of oGHRs and oPRLRs.

Materials and Methods

Materials

Recombinant oPL, oPRL and oGH were prepared as described previously (8, 13, 22). Purified recombinant hGM-CSF was a gift from Immunex Corp. (Seattle, WA). Plasmids encoding full-size oGHR-CFP and oPRLR-YFP in pcDNA3 expression vectors (Invitrogene Co., Leek, The Netherlands) were constructed as described previously (21). Preparation of hGM-CSFR α- and β-subunits, fused inframe with the TM and intracellular domains of oGHR and the long form of oPRLR: Namely hGM-CSFR-α-l-oPRLR (α-PRLR), hGM-CSFR-β-l-oPRLR (β-PRLR), hGM-CSFR-α-oGHR, and hGM-CSFR-β-oGHR in pECE was as described previously (20). OPTIMEM was purchased from Life Technologies, Inc. (Gaithersburg, MD). Fetal bovine serum was purchased from Sigma (St. Louis, MO) and Fugene 6 from Roche Diagnostics Co. (Indianapolis, IN). AL47 anti-GHR, a rabbit polyclonal serum raised against a bacterially expressed N-terminal His-tagged fusion protein incorporating hGHR residues 271 to 620, and AL33 anti-Janus kinase (JAK)2, a rabbit polyclonal serum raised against a glutathione-agarose affinity-purified glutathione-S-transferase fusion protein incorporating murine JAK2 residues 746-1129, were received from Dr. S. J. Frank. and goat anti-PRLR S46 was prepared in our laboratory (23). Molecular weight markers for SDS-PAGE, DMEM, and DMEM-HAM F12 medium were obtained from Life Technologies, Inc. SDS-PAGE reagents were purchased from Bio-Rad Laboratories, Inc. (Hercules, CA). Fetal calf serum was purchased from Boehringer (Mannheim, Germany). Other materials were from Sigma.

Cell culture and stable transfection

CHO cells were grown in HAM-F12 medium containing 10% fetal calf serum and maintained at 37 C in a humidified atmosphere gassed with 95% air, 5% CO_2. GC3 minimal medium used before hormonal induction experiments was composed of DMEM-F12, supplemented with glutamine, 100 μg/ml penicillin/streptomycin, nonessential amino acids, and transferrin without insulin for MAPK studies or insulin for others. To produce stable chimeric receptor clones, CHO cells were cotransfected with the pSV2-Neo vector and α-PRLR-pECE, β-PRLR-pECE, α-GHR-pECE, β-GHR-pECE eukaryotic expression plasmids (20). All transfections were carried out using ExGen 500 (Euromedex, Souffelweyersheim, France) according to the manufacturer's protocol. To get the same probability of plasmid integration, we cotransfected cells with the same amount of each construct. Neomycin (G418)-resistant clones were isolated over a period of 1 wk and amplified under 500 μg/ml G418 selection for 1 month. Chimeric homodimer (α-PRLR/β-PRLR and α-GHR/β-GHR)- and heterodimers (α-GHR/β-PRLR)-expressing clones were screened for ^{125}I-hGM-CSF binding and signal transducer and activator of transcription (STAT)5-DNA-binding activity. The hormone labeling and binding experiments were performed according to the protocol described for PRL by Goupille *et al.* (23). Expression of the transcripts was finally evaluated in each selected clone by RT-PCR with specific primers.

Total cell protein extracts and immunoprecipitation

Each stable CHO clone was grown to 80% confluence in 100-mm diameter plates before starving in a GC3 minimal serum-free medium one night before hormonal stimulation. For phosphorylation experiments, cells were treated with 50 ng/ml hGM-CSF for the indicated times: 5′ for receptor phosphorylation, 3′ and 7′ for JAK2 phosphorylation, and 15′ and 30′ for STAT activation. Then the cells were rinsed twice with PBS containing 1 mM sodium orthovanadate and scraped in 500 μl lysis buffer [1% vol/vol Brij 96 in 20 mM Tris-HCl (pH 8), 137 mM NaCl, 2.7 mM KCl, 10% vol/vol glycerol] containing protease and phosphatase inhibitors (10 mM benzamidine, 10 mM NaF, 2 mM EGTA, 5 mM NaPP, 20 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM phenylmethylsufhonyl fluoride, and 2 μg/ml each of leupeptin, pepstatin, and aprotinin). After lysis on a rotating wheel for 30 min at 4 C, the insoluble material was removed by centrifugation for 10 min at 12,000 $\times$ *g*. Immunoprecipitation was then carried out by overnight incubation of the supernatant containing the solubilized proteins with 10 μl S46 anti-PRLR antibody, 2 μl AL47 anti-hGHR serum, or 2 μl AL33 anti-JAK2 serum. The immune complexes were harvested in rotating tubes with protein G-Sepharose (Sigma) for another 2 h at 4 C. The beads were then recovered by a brief centrifugation at 8000 $\times$ *g*, and resuspended in 1.5 ml washing buffer [20 mM Tris-HCl (pH 8), 137 mM NaCl, 2.7 mM KCl, 0.1% vol/vol Triton X-100]. This washing step was repeated three times, and the pellets were boiled for 5 min in 50 μl Laemmli buffer [50 mM Tris-HCl (pH 6.8), 10% vol/vol β-mercaptoethanol, 5% sodium dodecyl sulfate (SDS), 21% vol/vol sucrose, and bromophenol blue] before loading to an 8% SDS-polyacrylamide gel.

FRET analysis

Human embryonic kidney (HEK)-293T cells were cultured at 37 C in 5% CO_2 in DMEM containing 10% fetal bovine serum. For transient transfections, cells were grown on coverslips in a 12-well culture dish (1.2×10^5cells/well). Each well was transfected with 400 ng GHR-CFP- and 400 ng PRLR-YFP-encoding plasmids. DNA was premixed with 50 μl OPTIMEM and 3 μl Fugene 6 reagent. The mixture was incubated at room temperature for 30 min and added to the cells. After 20 h of incubation, the cells were washed with PBS, and serum-free DMEM was added for 7 h. Then 400 ng of respective hormone (diluted in 0.1% BSA

in PBS) was added to each well for a different incubation time. Incubation was terminated by washing the cells twice with PHEM (60 mM Pipes, 25 mM HEPES, 10 mM EGTA, 2 mM $MgCl_2$, pH 7.5) buffer and fixing with 4% paraformaldehyde. The fixed cells were mounted using n-polyvinyl alcohol containing n-propyl galeate in PBS. Cells were analyzed by wide-field microscope (Olympus Corp., Tokyo, Japan). Fluorescence cubes were fitted with filters (Chroma Technology Corp., Brattlebough, VT) in the following configuration: CFP, Ex 425 nm/40, Dm 460 nm, Em 495 nm/20; YFP: Ex HQ525 nm/10, Dm 535 nm, Em HQ 560 nm/40; and FRET: Ex 425 nm/40, Dm 460 nm, Em HQ560 nm/40.

FRET was determined as described previously (24). Briefly, excitation of donor (GHR-CFP) and acceptor (PRLR-YFP) was performed with the CFP filter set and the YFP filter set, respectively. The FRET filter set was used to obtain both the donor and acceptor fluorescence. According to Gordon *et al.* (24), we modeled the fluorescent signal as follows: Dd (signal from cells expressing GHR-CFP using the CFP filter set), Fd (signal from cells expressing GHR-CFP using the FRET filter set), Ad (signal from cells expressing GHR-CFP using the YFP filter set), Da (signal from cells expressing PRLR-YFP using the CFP filter set), Fa (signal from cells expressing PRLR-YFP using the FRET filter set), Aa (signal from cells expressing PRLR-YFP using the YFP filter set), *df* (signal from cells expressing GHR-CFP and PRLR-YFP using the CFP filter set), Ff (signal from cells expressing GHR-CFP and PRLR-YFP using the FRET filter set), and Af (signal from cells expressing GHR-CFP and PRLR-YFP using the YFP filter set). With these data the FRET data were corrected for cross-talk and filter leaks as well as potential differences in the concentrations of the donor and acceptor. A background (region with no cells) was subtracted from the foreground value (region within the cell). Approximately nine images were taken from each specimen using a CCD camera (C4742–98, Hamamatsu Corp., Hamamatsu City, Japan), six to seven regions on cell membranes were chosen from each image, and then fluorescence intensity was quantified using Universal Imaging's Metamorph program. To verify the FRET observed with this sensitized emission approach, acceptor photobleaching was performed in some cases using the LSM 510 confocal microscope (Carl Zeiss, Thornwood, NY). Bleaching was performed with the laser set at 514 nm and at maximum power for 50 iterations. Fluorescent intensities of regions of interest were obtained with LSM software, and data analysis was performed with Excel (Microsoft Corp.).

Preparation of nuclear extracts for EMSA

Stable chimeric CHO clones were treated with 50 ng/ml hGM-CSF, rinsed with cold PBS, and scraped into extraction buffer [20 mM Tris-HCl (pH 8), 137 mM NaCl, 2.7 mM KCl, and 10% glycerol] containing protease and phosphatase inhibitors. Lysis was performed by adding Nonidet P-40 at a final concentration of 0.5% (vol/vol) to the cell suspensions. After homogenization and a brief incubation at 4 C for 5 min, the mixtures were carefully layered onto 6 ml separation buffer A [10 mM HEPES (pH 7.7), 25 mM KCl, 2 mM EDTA, 0.5 mM EGTA (pH 8), containing 1 M sucrose]. After a 15-min centrifugation at 6500 × *g*, the nuclear pellets were resuspended in buffer B [20 mM HEPES (pH 7.7), 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% glycerol]. The nuclear factors were extracted by addition of 4 M NaCl (one tenth of the final volume), and after 30 min gentle mixing at 4 C on a rotating wheel, the precipitated material was removed by a 30-min centrifugation at 12,000 rpm. Finally nuclear extracts were rapidly frozen at −80 C until use. Proteins in nuclear extracts were quantified with a BCA protein assay kit (Pierce Chemical Co., Bezons, France) according to the manufacturer's protocol with BSA as a standard.

Western blot analysis

Extracted proteins were separated overnight at 60 V by 8% or 12% SDS-PAGE (acrylamide:bis-acrylamide 29:1), respectively, for STAT and MAPK detection. Electrotransfer to 0.22-μm nitrocellulose was carried out at 400 mA for 4 h in Laemmli buffer containing 20% methanol. Membranes were stained, washed, and incubated overnight at 4 C under agitation with a primary antibody diluted in 5% nonfat milk Tris-buffered saline [10 mM Tris-HCl (pH 7.5), 150 mM NaCl, containing 0.1% vol/vol Tween 20]. The following antibodies were used: monoclonal mouse antiphospho-tyrosine 4G10 antibody at 1:2000 dilution (UBI, Lake Placid, NY), goat anti-PRLR S46 serum at 1:2000, polyclonal rabbit anti-hGHR AL47 at 1:2000, polyclonal rabbit anti-STAT1, STAT1P, STAT3, STAT5, STAT5P at 1:1000 (Euromedex), monoclonal mouse anti-STAT3P (Euromedex), polyclonal rabbit anti-MAPK, antiphospho-MAPK at 1:1000 (New England Biolabs, Inc., Boston, MA), antiphospho-JAK2 (UBI), and AL33 anti-JAK2 serum at 1:1000. Membranes were then washed and incubated with a secondary antibody coupled to horseradish peroxidase (antimouse at 1:2,000, antigoat at 1:20,000, and antirabbit at 1:15,000) for 45 min to 1 h at room temperature. After washing, the targeted proteins were identified by enhanced chemiluminescence (ECL reagent, Amersham, Orsay, France). For reprobing, blots were treated with stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl, pH 6.8) at 60 C for 30 min and washed overnight in Tris-buffered saline 0.1% Tween-20 before incubation with another primary antibody.

RNA preparation and RT-PCR analysis

Cells were lysed in Trizol reagent (Invitrogen SARL, Cergy Pontoise, France), and total RNA was harvested according to the manufacturer's protocol. Total RNA (3 μg) was used as a template for the RT-PCR using random hexamers and Superscript Reverse Transcriptase II (Invitrogen). Reactions were performed according to the manufacturer's protocol. For reverse transcription, 5 μl of the 1:2 diluted reverse transcription products were amplified by PCR. Four specific primers were used for the PCR experiments: GGGCCCCTGCAGGTCGACATGCTTCTCCTGGTAACAAGC (α-hGM-CSFR 5′primer), GGGCCCCTGCAGGTCGACATGGTGCTGGCCCAGGGGCTG (β-hGM-CSFR 5′ primer), GAATTCAAGCTTTCTAGACTACGGCATGATTTTGTTCAG (oGHR 3′ primer), and GAATTCAAGCTTTCTAGACTAAGGCAGGGCTGGCGG (oPRLR 3′ primer). The four cDNAs corresponding to the four chimeric receptors (α-PRLR, β-PRLR, α-GHR, and β-GHR) were then detected on a 0.8% agarose gel at, respectively, approximately 2010 bp, approximately 2350 bp, approximately 2080 bp, and approximately 2420 bp.

EMSA

Nuclear extracts from various stable chimeric CHO clones treated for 15 min with hGM-CSF (50 ng/ml) were prepared as described, and an EMSA was performed. A complementary synthetic oligonucleotide corresponding to the sequence 5′-GAGAATTCTTAGAATTTAAA-3′ derived from the rabbit αS1-casein promoter (−104 to −85 region) was used as a probe. This sequence, encompassing the STAT5-binding site was end-labeled with γ^{32}ATP (4500 Ci/mmol) and T4 polynucleotide kinase as previously described (25). Briefly, 3 μg nuclear extracts were mixed in a 10-μl reaction containing 10 mM HEPES (pH 7.6), 50 mM KCl, 8% vol/vol Ficoll, 0.2 mM EDTA, 0.5 mM DTT, and 1 μg poly(dI-dC) (Pharmacia, Uppsala, Sweden). After a 30-min preincubation at room temperature, the probe was added (50,000 cpm) and then incubated for a further 30 min. Free DNA and DNA-protein complexes were separated by electrophoresis in a 6% acrylamide:bisacrylamide (38:2) gel in 0.25 × Tris-borate EDTA (22 mM Tris, 22 mM borate, 0.6 mM EDTA, pH 8) at 200 V. Gels were then dried and analyzed by autoradiography.

Results

FRET analysis in transiently transfected HEK-293T cells

Previous experiments have documented that YFP- and CFP-tagged oPRLRs and oGHRs retain their full ability to activate the STAT5-mediated expression of the LUC reporter gene in transiently transfected HEK-293T cells (21). This model was therefore used in the present study to detect FRET between oPRLR-YFP and oGHR-CFP. FRET involves the transfer of energy from an excited fluorophore (donor) to a second fluorophore (acceptor) in a nonradiative manner. Thus, when exciting the donor at the proper wavelength, the acceptor produces an emission and donor emission is decreased. Although YFP and CFP are commonly used pairs, the fluorophores possess some overlapping spectra, making separation of YFP emission from CFP emission with standard filters difficult (cross-talk). The amount of cross talk cannot

be measured directly in cells expressing both YFP and CFP. It can, however, be determined in cells expressing either YFP or CFP alone. Therefore each cotransfected specimen was examined together with its single-transfected counterparts. Figure 1 shows fluorescent images of HEK-293T cells transfected with PRLR-YFP-, GHR-CFP-, and PRLR-YFP/GHR-CFP-encoding plasmids. Each specimen was examined through three filter sets as described in *Materials and Methods*. Images show cross-talk events: GHR-CFP, excited at 425 nm, leaks through the FRET emission filter set (Fd), and PRLR-YFP is directly excited at 425 nm when imaged through FRET filter set (Fa). However, the use of a previously described method allowed us to detect corrected FRET (24). It should be noted that the localization pattern of the fused protein appeared to be confined to both cell membrane and the nuclear regions.

To study the effect of oPL on GHR-CFP and PRLR-YFP heterodimerization, FRET between CFP and YFP was measured after hormone stimulation. To capture the initial effect of oPL stimulation, cells were stimulated with oPL for short periods and immediately washed and fixed. The FRET values at each time point collected from three different experiments are shown in Fig. 2A. Before oPL stimulation, a low FRET value was calculated, indicating low energy transfer. Soon after stimulation the FRET value was significantly elevated reaching a maximal level after 2.5 to 3 min and indicating proximity between the two fluorophores. This elevation was, however, transient, lasting about 1 min, and over longer oPL stimulation periods FRET values decreased to the initial value (two experiments) or a slightly elevated value (one experiment). To verify the FRET between oGHR-CFP and oPRLR-YFP, the method of donor (CFP) emission recovery because of acceptor (YFP) photobleaching was also applied (26). Cotransfected cells, stimulated for 3 min by oPL, were fixed as described above, and the fluorescence intensity of CFP was measured before and after photobleaching of YFP by excitation at 514 nm, using an LSM 510 confocal microscope (Zeiss). Photobleaching increased the mean intensity of CFP fluorescence by 22.5%, whereas nonstimulated cells showed a slight decrease. The specificity of oPL action was further demonstrated by the fact that oGH and oPRL that could respectively homodimerize oGHR-YFP and oGHR-CFP or oPRL-YFP and oPRL-CFP (not shown) did not induce any elevations in FRET in cells transfected with oGHR-CFP and oPRLR-YFP, indicating that only oPL induced heterodimerization of oPRLRs and oGHRs (Fig. 2B).

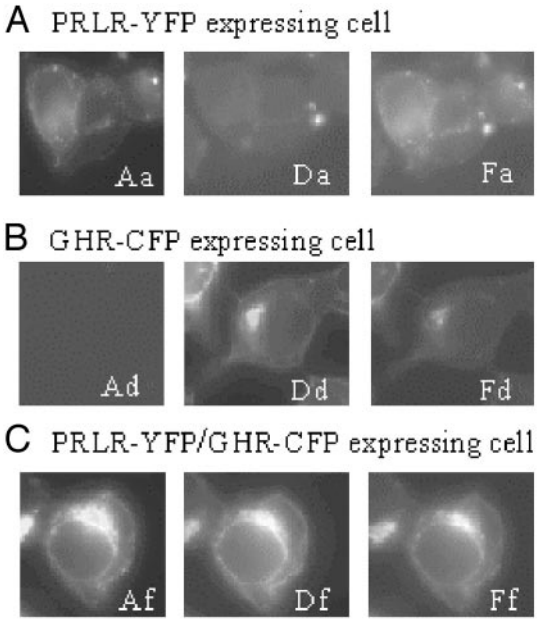

FIG. 1. Fused-protein expression in HEK-293T cells. Fluorescent images of HEK-293T cell expressing oPRLR-YFP (A), oGHR-CFP (B), and oPRLR-YFP/oGHR-CFP (C). Each image was taken with YFP (*left panels*), CFP (*center panels*), and FRET (*right panels*) filter sets, as described in *Materials and Methods*.

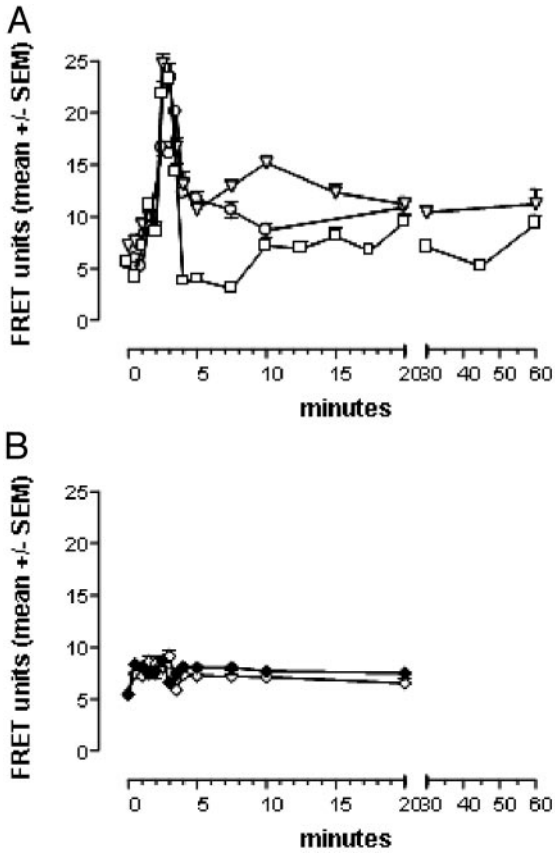

FIG. 2. Time-dependent oPL induction of oPRLR/oGHR heterodimerization. Transiently transfected HEK-293T cells, expressing oGHR-CFP and oPRLR-YFP, were stimulated with oPL (A, three experiments: 127, △, ○, □), oPRL (♦), or oGH (◇) (B). At various time points, the cells were washed of hormone and fixed. Mean FRET (mean ± SEM) was calculated as described in *Materials and Methods*. Each time point represents a mean of 40–45 regions of interest obtained at various times after stimulation and 69 regions of interest for time 0 (before the stimulation).

Functional analysis of stable CHO clones transfected with homodimeric α-PRLR/β-PRLR, α-GHR/β-GHR, or heterodimeric α-GHR/β-PRLR

Because oPL can homodimerize oPRLRs and heterodimerize oPRLRs and oGHRs, it is impossible to distinguish between the signaling resulting from either homodimerization or heterodimerization in cells transfected simultaneously

with both types of receptors. Therefore, to investigate the uniqueness of the signals, we developed an alternative model based on three stable CHO clones transfected with the four available chimeric hGM-CSFR constructs described above. Because a high-affinity and functional receptor for hGM-CSF can only be obtained by the sequential association of one α- and one β-subunit of hGM-CSFR, signal transduction induced in stable clones transfected with two constructs presenting both subunit ECDs can only be attributed to ligand-induced α/β association. In the present study, we cotransfected α-GHR and β-PRLR constructs for the production of a heterodimeric clone because this has been shown to give better transcription activation than the reciprocal association (20). Choice of those constructs resulted also from the finding that oPL sequentially binds oGHR through site 1 and then oPRLR through site 2.

In a first-selection step, we screened our clones for best binding capacities using ^{125}I-hGM-CSF. Because it is well established that the α-chain of GM-CSFR confers low-affinity GM-CSF binding without any transduction signal, whereas the β-chain does not bind GM-CSF by itself but confers high-affinity binding when associated with α-chain (27), the binding experiments were not sufficient to ensure that all selected clones had both GM-CSFR subunits. Therefore, clones chosen for best binding capacities were screened for their STAT5-DNA-binding activity using EMSA (Fig. 3A) and tested by RT-PCR for expression of chimeric receptors. All expressed the correct-size mRNA (Fig. 3B). The three selected chimeric clones, namely, 25, 42, and 52, expressing, respectively, homodimeric α-PRLR/β-PRLR, α-GHR/β-GHR, and heterodimeric α-GHR/β-PRLR associations, were thus chosen for further studies.

To verify the expression of the transfected genes, clones 25, 42, and 52 were also characterized by immunoprecipitation followed by Western blotting (Fig. 4). Chimeric α-GHR and β-GHR are detected in clone 42 at the expected size of approximately 120 kDa, α-PRLR, and β-PRLR are detected at approximately 100 kDa in clone 25, and α-GHR and β-PRLR are detected in clone 52. Functionality of those proteins was verified by 5-min hGM-CSF-induced phosphorylation of both PRLRs and GHRs that clearly took place on hormonal stimulation, demonstrating that the chimeric model is well suited to our investigation. Further evidence of hGM-CSF-induced heterodimerization can be attributed to the finding that both PRLRs and GHRs (in clone 52) could be detected in extracts immunoprecipitated by both S46 anti-PRLR and AL47 anti-GHR antibodies with an almost equal efficiency (Fig. 4). Because JAK2 recruitment is not the same between these two kinds of associations (constitutively associated to PRLR and recruited in the case of GHR), we also tested JAK2 activation in all three chimeric clones. JAK2 phosphorylation can be seen very early after hGM-CSF stimulation as observed on the kinetics shown in Fig. 5, but no obvious differences seem to appear between homodimeric and heterodimeric associations.

Determination of STAT activation and MAPK activity in homodimeric and heterodimeric CHO clones

To elucidate whether the heterodimerization of oPRLR and oGHR leads to a unique signal, different from that produced by the homodimerization of GHRs or PRLRs, we tested two well-established effects of PRLR and GHR signaling, namely STAT1, STAT3, and STAT5 activation (Fig. 6) and (ERK1/2) MAPK activation (Fig. 7), both of which play central roles in differentiation and proliferation mechanisms. Each experiment was repeated three times, and the representative blots are shown.

As shown in Fig. 6, upon hormonal activation all three STATs were phoshorylated in cells expressing homodimeric α-PRLR/β-PRLR and α-GHR/β-GHR, confirming what is known for full-length receptors. The present results do not

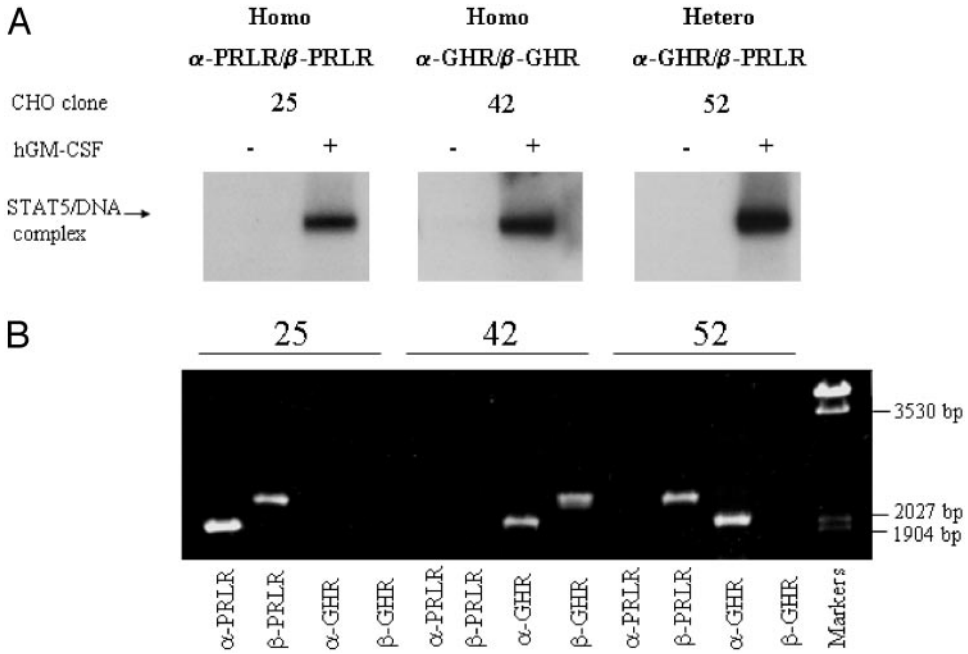

FIG. 3. Characterization of three stable chimeric hGM-CSFR CHO clones. CHO cells stably transfected with the following different chimeric receptors (α-PRLR/β-PRLR, α-GHR/β-GHR, and α-GHR/β-PRLR) were prepared and tested for EMSA experiment showing STAT5-DNA-binding activity for the three selected CHO clones (A) and expression chimeric receptors' mRNA demonstrated by RT-PCR for each selected clone (B). Results show α-GHR cDNA at approximately 2080 bp, α-PRLR cDNA at approximately 2010 bp, β-GHR cDNA at approximately 2420 bp, and β-PRLR cDNA at approximately 2350 bp.

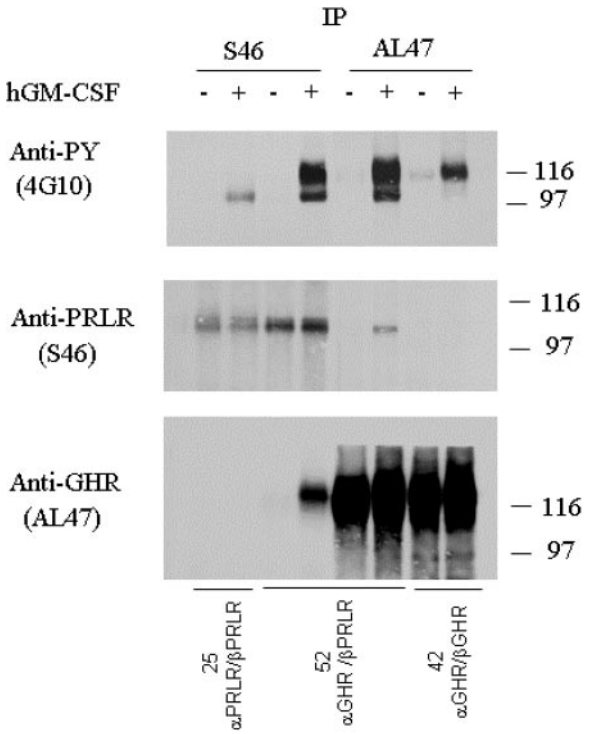

FIG. 4. Receptor expression and functionality of three stable chimeric hGM-CSFR CHO clones. Total cell extracts from homodimeric α-PRLR/β-PRLR, α-GHR/β-GHR, and heterodimeric α-GHR/β-PRLR clones treated or not with 50 ng/ml hGM-CSF for 5 min were immunoprecipitated overnight with S46 anti-PRLR or AL47 anti-GHR and loaded onto an 8% SDS-polyacrylamide gel for Western blot analysis. Proteins were then transferred to nitrocellulose and immunoblotted with S46, AL47, or 4G10 anti-phosphotyrosine antibody, and immune complexes were revealed as described in *Materials and Methods.* Chimeric α-PRLR and β-PRLR appear as a band at approximately 100 kDa, and chimeric α-GHR and β-GHR as a band at approximately 120 kDa.

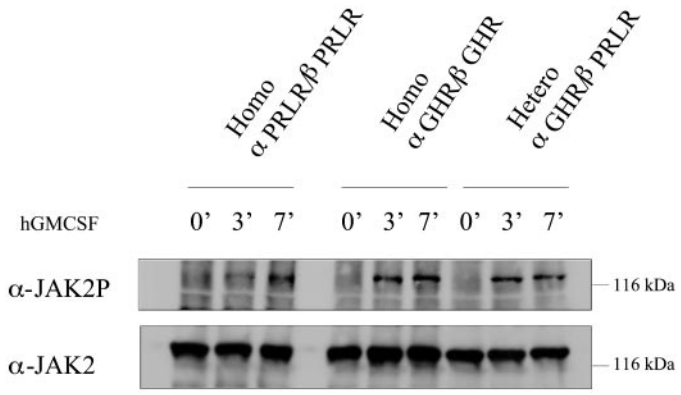

FIG. 5. JAK2 expression and phosphorylation in homodimeric and heterodimeric CHO clones. All three CHO clones were harvested and stimulated with 50 ng/ml hGM-CSF for 0, 3, and 7 min. Solubilized total protein extracts were prepared as previously described in *Materials and Methods* and immunoprecipitated with 2 μl anti-JAK2 (AL33) antiserum. Proteins were then loaded onto an 8% SDS-polyacrylamide gel, blotted, and revealed using an antiphospho-JAK2 (UBI) and anti-JAK2 (AL33) antiserum at 1/1000e dilution. JAK2 protein appears as a band migrating at approximately 120 kDa.

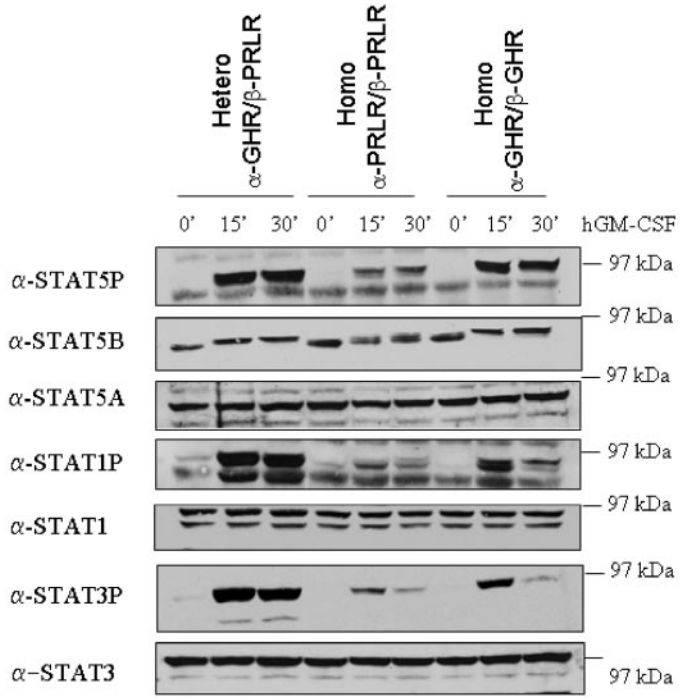

FIG. 6. Comparison of STAT recruitment and activation between stable homodimeric and heterodimeric CHO clones. Nuclear extracts from homodimeric α-PRLR/β-PRLR (clone 25), α-GHR/β-GHR (clone 42), and heterodimeric α-GHR/β-PRLR (clone 52) clones treated or not treated with 50 ng/ml hGM-CSF for 15 and 30 min were loaded onto an 8% SDS-polyacrylamide gel. Western blot analysis was then performed using α-STAT1, α-STAT3, α-STAT5A, α-STAT5B, α-STAT1P, α-STAT3P, and α-STAT5P antibodies. STAT proteins appear as approximately 92- and approximately 95-kDa bands. Stripping and reprobing were performed as described in *Materials and Methods*.

enable an absolute distinction between activation of STAT5A and -B because antiphosphorylated STAT5 antibody recognizes both forms. The shift in STAT5B is due to serine phosphorylation of STAT5B and results in overlapping migration of STAT5A and B. Thus, it is likely that both STAT5A and -B are activated. The results clearly demonstrate that the heterodimeric association of α-GHR and β-PRLR is also capable of activating STAT1, STAT3, and STAT5, but the kinetics of phosphorylation is different. This difference can be evidenced by comparing the extent of phosphorylation of different STATs in the same cells at 15 and 30 min. For this purpose we have scanned the phosphorylated bands (Fig. 6) using nonsaturated autoradiographs and normalized them by correcting for background and total STATs' expression. Then we have calculated the ratios between the phosphorylated STATs after 30 and 15 min of hGM-CSF stimulation and summarized those results in Table 1. It is evident that the ability to phosphorylate the three STATs differs between the homodimeric and the heterodimeric associations. In the case of the heterodimerization of α-GHR and β-PRLR, STAT5 activation did not notably change between 15′ and 30′ of stimulation (−14%), and STAT1 and STAT3 phosphorylation are decreased by 30%. For the homodimeric α-PRLR/β-PRLR association, STAT5 phosphorylation was also preserved to a similar extent (−18% change), whereas STAT1 and STAT3 phosphorylations were remarkably lower (Table 1 and Fig. 5). This difference in STAT kinetics was even more pronounced in the case of the homodimeric α-GHR/β-GHR

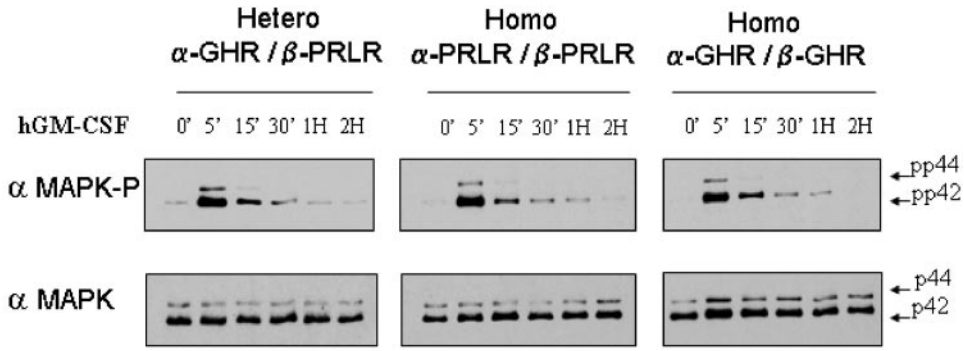

FIG. 7. Time course of MAPK (ERK1/2) activation in stable homodimeric and heterodimeric CHO clones. Three stable chimeric receptor α-GHR/β-GHR, α-PRLR/β-PRLR, and α-GHR/β-PRLR CHO clones were incubated in serum-free medium without insulin overnight before treatment with hGM-CSF (50 ng/ml) for 0′, 5′, 15′, 30′, 1 h, and 2 h. Western blot analysis was performed on 20 μg total cell extracts, using α-MAPK and α-MAPKP antibodies. Positions of ERK1/2 and phosphorylated ERK1/2 are indicated with *arrows* as 42- and 44-kDa proteins.

TABLE 1. Comparison of STATs phosphorylation at 15 and 30 min after hGM-CSF stimulation (50 ng/ml) in homodimeric and heterodimeric associations

Clone tested	Ratio of band intensity at 30/15 min[a]		
	STAT1	STAT3	STAT5
25 (α-PRLR/β-PRLR)	0.54	0.30	0.82
42 (α-GHR/β-GHR)	0.33	0.14	0.68
52 (α-GHR/β-PRLR)	0.70	0.70	0.86

[a] Quantitative analysis was performed using Aida software and Fujifilm camera apparatus. Normalization was made using nonsaturated autoradiographs, corrected for background and total STATs expression.

association, where STAT1-P and STAT3-P dramatically declined by 67% and 86%, respectively (Table 1 and Fig. 6).

In addition to STAT activation, we also investigated the mitogenic pathway by quantification of MAPK phosphorylation 5 min to 2 h after hGM-CSF stimulation (Fig. 7). The results demonstrate that both p44 ERK1 and p42 ERK2 MAPK were activated not only in the homodimeric α-PRLR/β-PRLR and α-GHR/β-GHR clones but also in the heterodimeric α-GHR/β-PRLR clone. However, in contrast to the activation of STAT1 and 3, no significant differences in the kinetics of MAPK activation could be detected between the homodimeric clones 25 and 42 and the heterodimeric clone 52.

Discussion

Lack of a specific receptor for oPL prompted us to investigate the putative way in which this hormone's signal is initiated in homologous systems (for review see Ref. 28). In addition to oPL's ability to substitute for PRL by homodimerizing oPRLRs (28), we speculated that there must be another specific means of oPL signaling that results from oPL-induced heterodimerization of oPRLRs and oGHRs. An additional argument for the unique action of oPL comes also from molecular evolution studies. Sheep and cows diverged 18×10^6 years ago and ruminant PLs evolved from duplication of the PRL gene (29). The rate of molecular evolution of ruminant PLs was extremely fast, as evidenced by the very high ratio of nonsynonymous to synonymous mutations, compared with the parallel changes in evolution of PRLs and GHs at the same period (29). Such a phenomenon can be interpreted as the incorporation of an adaptive rather than neutral mutation or as optimizing a novel function. Our present data support the notion that such a function is heterodimerization of ruminant PRLRs and GHRs leading to unique, albeit mostly unknown, physiological consequences. To prove this hypothesis, we first engineered soluble ECDs of both receptors and by several independent methods documented their oPL-induced heterodimerization (20). Then to prove that such heterodimerization is functional and cross-talk between the intracellular domains of the oGH and oPRL receptors is possible, we also prepared chimeric receptors consisting of intracellular and TM portions of oGHR or oPRLR and ECDs of hGM-CSFRα or -β and documented that on stimulation by hGM-CSF, the heterodimerized receptors are capable of transducing biological signal (20). However, direct evidence that oPL-induced heterodimerization of oPRLRs and oGHRs occurs in living cells, as well as the demonstration that such an event leads to signaling that is different from that resulting from homodimerization of oGH or oPRL receptors, was missing. To meet this challenge, in the present work we prepared FP-tagged oPRL and oGH receptors and proved their functionality (21).

The present study is the first demonstration that full-length oPRLRs and oGHRs associate under oPL activation in living cells. FRET methodology allowed us to show that on addition of saturating amounts of oPL (400 ng/well), the heterodimerization reaches its peak in 2.5–3 min, is specific to oPL, and is relatively transient and does not indicate significant existence of preformed dimers before hormonal stimulation (Fig. 2). The observed decrease in the FRET signal may result from either dissociation of the two receptors or from disappearance of the signal because of internalization. The present results do not enable us to distinguish among these phenomena and further investigation is required, although the coimmunoprecipitation experiments (see Fig. 4) hint that the chimeric heterodimer is internalized and at least partially preserved even after 5 min. Assuming the internalization is a slower process (30) and the half-life of the heterodimer of oGHR-ECD and oPRLR-ECD is 0.92 min (20), we attempted to calculate the half-life of the heterodimerized receptors in the membrane of the living cells on the basis of data presented in Fig. 2 in the range of 3–5 min. The average value of the three experiments was 1.05 ± 0.21 min (mean ± SEM), close to the value found for the soluble proteins in Biacore experiments (20).

To study the unique functional consequences of heterodimerization, we could not use oPL because in cells transfected with both GHRs and PRLRs, exposure to oPL leads to both homodimerization of oPRLRs and heterodimerization of oPRLRs and oGHRs. Therefore, we used chimeric recep-

tors that can be heterodimerized on exposure to hGM-CSF. We chose α-oGHR and β-oPRLR because hGM-CSF is known to bind sequentially to the α-ECD and β-ECD (27), thus simulating the sequential binding of oPL to GHRs and PRLRs (20). The advantage of our model is that all our constructed chimeric receptors had identical ECDs (hGM-CSFR α- and β-subunits) and thus most probably had the same association and dissociation kinetics with the ligand. Therefore, the observed functional consequences in terms of signal transduction strictly reflected the nature of the cytoplasmic domains present in homo- or heterodimeric associations.

We clearly demonstrated that α-oGHR and β-oPRLR associate and are phosphorylated on hGM-CSF stimulation, as evidenced by immunoprecipitation by both S46 anti-PRLR and AL47 anti-hGHR antibodies and exhibit almost equivalent efficiency (Fig. 4). Because the strong association between α-GHR and β-PRLR is maintained after immunoprecipitation and the cytosolic parts of the chimeric receptors are phosphorylated, they appear to be a very good tool for investigating and comparing transduction resulting from either homodimeric or heterodimeric associations. The results documented that hGM-CSF-induced homo- and heterodimerization of chimeric receptors α-GHR and β-PRLR were functional in the sense that they could both activate phosphorylation of STATs and MAPK-dependent pathways. However, whereas no or little differences were found in the kinetics of activation of STAT5 or MAPK, heterodimerization resulted in a prolonged phosphorylation of STAT1 and STAT3, compared with homodimerization of PRLRs and GHRs. Therefore our results suggest that the heterodimeric association of intracellular domains of oGHR and oPRLR is able to transduce the signal in a way that is kinetically distinct from that occurring on homodimeric associations.

It should be noted that the duration of the STAT activation signal might influence the specificity of cytokine signaling (31, 32). Work by Chang *et al.* (33) indicated that the recruitment of signaling molecules necessitates pairing of tyrosines on both intracellular receptor chains. In our work, we indicate that oPL is able to associate intracellular domains of a different nature and consequently, the pairing in *trans* of tyrosine residues located on each of identical molecules (effective in homodimers) does not occur. Moreover, conformational changes induced by heterodimeric interactions may also change the recruitment specificity of signaling molecules. This is probably one reason STAT recruitment in the case of heterodimers may be different. Although our present results do not provide the mechanism responsible for this difference, in view of the fact that neither oPRL nor oGH receptors possess the consensus sequence for binding STAT1 (YXPQ or YDXXH) or STAT3 (YXXQ), we assume that activation of STATs 1 and 3 may be a consequence of direct binding to phosphorylated Jak2 (34, 35), as was also shown in the case of gp130, which does not require receptor phosphorylation (36). To determine whether heterodimerization and homodimerizations affect differently JAK2 activation, phosphorylation of JAK2 was tested in the three clones expressing the α-oGHR/α-oGHR, β-oPRLR/β-oPRLR, and α-oGHR /β-oPRLR on hGM-CSF stimulation. Because no difference in JAK2 activation was detected (see Fig. 5), other mechanisms, such as inefficient recruitment of tyrosine phosphatases and/or SOCS proteins because of nonpairing of receptor tyrosines, are likely responsible for the different kinetics of STAT1 and -3 phosphorylation.

Our present findings raise the question of whether oPL-induced heterodimerization of oPRLRs and oGHRs plays any role in *in vivo* hormonal action, compared with the effects of GH or PRL. Results of such comparative studies are still very scarce. We have shown that oPL and oGH both have mammogenic effects in pseudopregnant ewes (37), and both stimulate the expression of uterine milk protein in ovariectomized ewes (38) and the growth of young lambs, but only the latter causes elevation of IGF-I levels (39). In another study (22), we documented that in contrast to oGH, oPL exhibits only a limited galactopoietic effect in lactating ewes, whereas no effect is obtained with oPRL. Others demonstrated the unique effect of oPL in pregnant ewes treated from d 101–107 of gestation with bovine GH or oPL, in which only the latter elevated expression of IGFBP3-I (40).

In conclusion, we suggest that in a homologous system oPL (and likely other ruminant PLs) simulates PRL action by homodimerization of PRLRs, but in addition it exhibits unique signaling resulting from heterodimerization of PRLRs and GHRs and mimics some but not all somatotrophic activities of oGH. The following step in our understanding of oPL's actions would be to determine specific gene activation patterns induced by homodimers or heterodimers in different target organs in which both kinds of receptors are expressed.

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